HEAT PROCESSING AND THE NUTRITIVE VALUE OF MILK AND MILK PRODUCTS [1]

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FIVE FIGURES

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The effect of heat processing on the nutritive value of food proteins has been the subject of extensive experimental work. The over-all viewpoint which persists, as a result of these investigations, is that proteins subjected to heat are not as well utilized for growth as are unheated proteins, and do not support favorable nitrogen balances. The explanation, however, for this deleterious effect of heat has not been completely elucidated. In recent years it has been suggested that the decrease in nutritive value observed with heat-damaged proteins is due to increased resistance to enzyme action. This resistance toward proteolytic enzymes apparently arises from the interaction of free amino groups with reducing sugars, producing the browning or Maillard reaction. This was recognized by Patton and Hill ('48), who found that tryptophan was destroyed by heating with glucose. Later, Patton, Hill and Foreman ('48) observed significant losses of lysine, arginine, tryptophan and histidine when casein was refluxed in a 5% solution of glucose for 24 hours at 96.5°C. These findings with casein were confirmed by other investigators, among whom were Henry, Kon, Lea and White ('48) and McInroy, Murer and Thiessen ('49).

[1] Preliminary reports of this work were presented before the Michigan Academy of Science at East Lansing, March 24, 1951, and the American Institute of Nutrition at Cleveland, April 30, 1951 (Schroeder, Iacobellis and Smith).

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The Maillard reaction has been found to occur also with commercial lactalbumin; Davis, Rizzo and Smith ('49) observed an extensive browning of this protein and a marked decrease in its nutritive values, as measured by rat growth, when it was autoclaved at 120°C. for 60 minutes. Using the same lot of protein, in a later study on nitrogen balance in dogs, Mader, Schroeder and Smith ('49) found that it gave a positive Molisch test. On extracting this protein with water, they were able to obtain a product which did not give a Molisch test and which, when heated and fed to dogs, gave a better digestibility and biological value than the heated, unextracted lactalbumin. They concluded, therefore, that the damage to the nutritive value of commercial lactalbumin was due to the interaction of lactose with the protein. Still later, Schroeder, Stewart and Smith ('51) observed that on autoclaving extracted lactalbumin with the same mole per cent of the reducing sugars, glucose, maltose, xylose and fructose, a definite decrease in the nutritive value of the protein was observed, strengthening further the hypothesis that the reaction between reducing sugars and proteins is responsible for the decreased nutritive value of heated proteins.

Aware of the possibility that such a reaction may occur in processed milk, the present investigation was undertaken first, to re-evaluate the possible nutritive damage to milk and milk products incurred during commercial heat processing, and second, to consider other factors which might influence the nutritive efficiency of the proteins of milk, such as the storage of frozen raw milk.

EXPERIMENTAL

Samples of raw milk, evaporated milk, and powdered milk were prepared at the station from the same raw milk supply.[2] The procedure which was followed in this preparation was as

[2] The authors acknowledge the help of Dr. H. L. Sipple of the Evaporated Milk Association and Mr. H. E. O. Heineman and Dr. E. A. Louder of the Pet Milk Company, Greenville, Illinois, for their aid in securing these samples of milk and milk products.

follows: 40,000 pounds of raw milk were pumped into a holding tank and agitated one hour before taking out the fluid unpasteurized milk samples, which were withdrawn in gallon containers and immediately packed in dry ice in an insulated refrigerator box. The original temperature of the raw milk when received at the above-specified station was 40°C. The analysis of the raw milk showed 3.73% fat and 12.22% solids. After collecting these raw milk samples for our study, the remaining volume of milk was moved through evaporated milk equipment, fore-warmed, condensed, homogenized, cooled and run into a cold storage tank. This condensed milk was analyzed for fat and total solids and then adjusted with water so that the final analysis showed 8.03% fat and 26.02% total solids. Immediately after adjusting the tank, the milk was passed through standard equipment and sterilized at 240°F. (10 lb.) for 15 minutes, representing the same procedure as is followed in the production of evaporated milk. Samples of milk were also collected from the condensed milk tank and dried in a spray box at a temperature of 188°F. (87°C.). Analysis of the milk powder showed 30.35% butter fat and 1.60% moisture. Samples of raw milk, evaporated milk and powdered milk derived from the same raw milk, as stated above, were shipped to our laboratory. In addition to these commercially processed milks, local raw milk was autoclaved at 10 and 15 pounds' pressure for 15 and 30 minutes in our own laboratory.

The composition of the protein-free basal diet, to which daily supplements of milk or milk reconstituted with water were added, is shown in table 1. Aliquots of fresh milk or reconstituted milk were mixed with this protein-free basal diet at such levels as to furnish the desired nitrogen intake, 160 to 200 mg protein nitrogen per kilogram of body weight per day. The total ration provided an optimum value of 58 to 77 calories per kilogram of body weight per day (Schroeder, Stewart and Smith, '51). The experimental subjects used in this investigation were 4 adult mongrel female dogs (9.6,

8.2, 7.3 and 12.5 kg), which were kept in standard individual metabolism cages with free access to water at all times.

The nutritive efficiency of the milk and the milk products was evaluated by the method of Murlin and associates ('41), which consists essentially in comparing the amount of urinary and fecal nitrogen of a subject fed a diet containing a "control" or reference milk, with the urinary and fecal nitrogen excretion of the same subject fed a similar diet but containing the experimental milk. In this study, the raw milk (control) was

TABLE 1

Control and experimental diets

CONSTITUENTS	AMOUNT
	%
Basal diet	
Sucrose	29.0
Dextrin	12.8
Crisco	15.0
Salt mixture (Wesson)	3.5
Bone ash	2.7
Milk supplement	
Protein (3.05–3.30%)	9.4
Fat (4.0%)	12.0
Carbohydrate (5%)	15.0
	99.4 [1]
Vitamin supplement	
ABDEC Kapseals (Parke Davis and Company)	

[1] Caloric equivalent, 5.15 calories/gram.

in most cases (see table 2) used in the basal diet for three days, followed by a 4-day period in which the commercially processed milk (experimental) was used, and this routine was repeated twice for each experimental period.

Urine samples were collected quantitatively and preserved with sulfuric acid and toluene, accurate collection being insured for each period by catheterization. After making to volume, aliquots were analyzed for nitrogen. The feces were separated for each period by carmine, then covered with eth-

TABLE 2

Food, urinary, and fecal nitrogen data

DIET	DAYS	TOTAL FOOD N Dog no. 1	2	3	4	TOTAL URINARY N Dog no. 1	2	3	4	TOTAL FECAL N Dog no. 1	2	3	4
			gm				gm				gm		
Basal + frozen raw milk	5	9.90	9.53	8.80	12.83	7.13	7.60	5.77	6.20	1.12	1.05	1.34	1.77
Basal + frozen raw milk	4	7.92	7.63	7.04	10.26	5.44	6.86	5.47	6.46	1.01	1.00	1.02	1.11
Basal + frozen raw milk	4	7.94	7.64	7.06	10.28	5.70	6.50	5.08	7.03	0.97	1.22	1.32	1.62
Basal + local raw milk	3	6.02	5.79	5.35	7.80	3.95	4.10	4.06	5.74	0.93	0.79	0.67	0.85
Basal + frozen raw milk	4	7.94	7.64	7.06	10.28	5.77	7.06	4.92	8.20	1.07	0.94	0.86	2.46
Basal + local raw milk	7	14.05	13.53	12.49	18.21	9.80	9.66	8.16	13.33	1.71	1.82	2.00	3.25
Basal + local raw milk	3	6.03	5.81	5.36	7.81	4.20	4.80	3.72	5.88	1.29	0.99	1.03	1.24
Basal + evaporated milk	4	7.97	7.68	7.08	10.33	4.04	5.63	4.76	7.58	0.50	0.40	0.62	0.77
Basal + local raw milk	3	6.04	5.82	5.37	7.83	4.13	4.69	3.80	5.23	1.52	1.04	0.93	1.89
Basal + evaporated milk	8	15.95	15.35	14.17	20.66	12.01	10.42	9.14	15.33	1.30	2.43	1.62	2.71
Basal + local raw milk	4	8.039	7.74	7.148	10.41	5.50	5.54	4.25	7.95	1.47	1.04	1.25	1.58
Basal + milk powder	3	5.959	5.74	5.30	7.719	3.64	4.08	3.78	5.74	0.63	0.60	0.49	1.48
Basal + local raw milk	7	14.08	13.56	12.519	18.248	8.06	11.48	7.93	13.06	2.69	1.92	2.12	2.56
Basal + milk powder	4	7.94	7.64	7.06	10.28	4.74	5.51	3.52	6.33	0.99	1.20	1.27	0.96
Basal + local raw milk	3	6.04	5.82	5.37	7.83	3.61	3.95	3.92	4.98	0.92	0.81	0.73	0.92
Basal + local raw milk	3	6.40	6.16	5.69	8.29	3.56	4.22	3.85	5.20	1.55	0.92	0.66	1.15
Basal + autoclaved raw milk	4	8.31	8.007	7.39	10.77	6.44	6.30	4.90	6.92	0.50	0.49	0.84	1.20
Basal + local raw milk	3	6.42	6.185	5.142	8.32	4.28	4.23	3.82	5.15	0.43	0.83	0.72	1.32
Basal + autoclaved raw milk	4	8.36	8.05	6.69	10.83	6.72	6.81	4.34	7.56	1.01	0.83	0.64	0.76
Basal + local raw milk	3	6.40	6.16	5.12	8.29	3.64	4.31	3.91	5.31	1.09	0.95	0.54	1.11
Basal + autoclaved raw milk	4	8.36	8.05	6.69	10.83	6.48	5.75	4.47	7.56	0.68	1.01	0.63	1.12
Basal + local raw milk	3	6.42	6.19	5.15	8.32	4.65	4.03	3.89	5.05	0.94	0.87	0.79	1.17
Basal + autoclaved local raw milk	4	8.39	8.08	6.72	10.87	6.55	6.34	4.31	7.71	0.90	0.917	1.34	1.40
Basal + local raw milk	3	6.42	6.19	5.15	8.32	4.32	4.36	3.73	5.01	1.01	0.93	0.92	1.39
Basal + autoclaved local raw milk	4	8.56	8.24	6.85	11.08	5.99	6.22	4.26	7.20	1.03	1.32	0.93	2.01
Basal + local raw milk	3	6.46	6.22	5.17	8.37	4.16	4.42	5.12	4.91	1.45	1.01	0.61	1.91
Basal + autoclaved local raw milk	4	8.61	8.29	6.89	11.15	6.25	6.72	4.11	7.02	1.61	0.77	1.09	2.20
Basal + local raw milk	3	6.46	6.22	5.17	8.37	4.11	4.39	5.03	5.07	1.29	1.07	0.44	1.24
Basal + frozen raw milk	4	8.10	7.80	6.49	10.50	5.74	6.13	4.51	5.48	1.47	0.45	0.55	1.46
Basal + local raw milk	3	6.46	6.22	5.17	8.37	4.31	5.46	3.97	4.99	0.96	1.50	0.77	0.96
Basal + frozen raw milk	3	5.92	5.71	4.74	7.67	4.22	4.99	3.47	4.72	0.58	0.49	0.80	1.11

anol containing 1% sulfuric acid, dried on a steam cone, ground, and aliquots analyzed for nitrogen. The macro-Kjeldahl method was used for analysis of the nitrogen and all determinations were made in duplicate.

Preliminary studies with a standard protein were run and it was observed that a diet containing 10% casein maintained the dogs in nitrogen balance. On this basis the necessary amount of nitrogen from the various types of milk was determined and the milk mixed with the basal diet so as to furnish the equivalent of approximately the 10% protein requirement. The added milk represented the sole protein nitrogen source. Nitrogen intake varied from 160 to 200 mg protein nitrogen per kilogram per day. All diets were isocaloric and the nitrogen intake was kept constant for each dog.

The averages of the nitrogen balances of the 4 dogs (expressed as milligrams N per day per dog), as they were alternately fed the control milk and the experimental milk, appear in figure 1 and should be considered with the detailed data shown in table 2. In the present investigation, pooled frozen raw milk was first used as the control milk. Since this was a protracted study, this frozen raw milk was compared to the raw milk from a local source.[3] Inasmuch as no difference in nutritive value was observed between these two milks, the local raw milk was then used as the control milk. In addition, the original frozen raw milk was kept in frozen storage for 6 months and at this time its nutritive index re-evaluated. Although there were slight variations, it is obvious that all the dogs were in positive nitrogen balance on all of the milk samples employed.

The nutritive value of a protein depends upon two factors, its digestibility and its biological value (see footnote, table 3). These two quantitative expressions of the nutritional efficiency of the protein of milk were determined by a modification of the method of Murlin and associates. In essence, the control milk was assigned a digestibility and biological value

[3] Kindly furnished by the Borden Company, Detroit, Michigan.

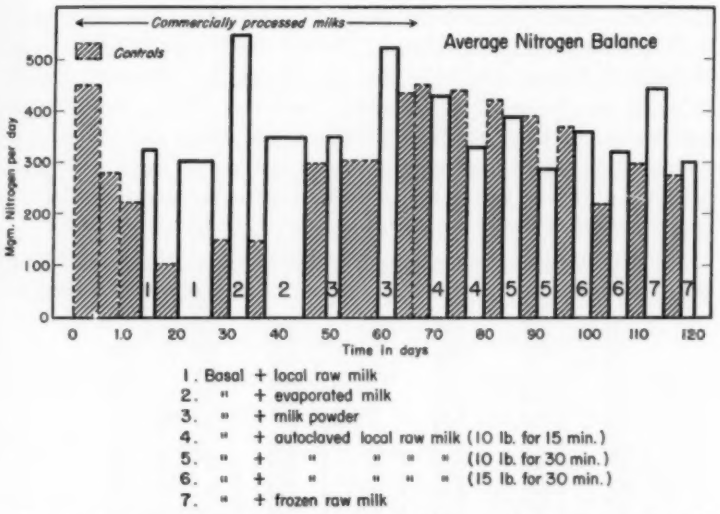
Commercially processed milks
Controls
Average Nitrogen Balance
Mgm. Nitrogen per day
500
400
300
200
100
0
1 1 2 2 3 3 4 4 5 5 6 6 7 7
0 10 20 30 40 50 60 70 80 90 100 110 120
Time in days
1. Basal + local raw milk
2. " + evaporated milk
3. " + milk powder
4. " + autoclaved local raw milk (10 lb. for 15 min.)
5. " + " " " " (10 lb. for 30 min.)
6. " + " " " " (15 lb. for 30 min.)
7. " + frozen raw milk

Fig. 1 Average nitrogen balance in dogs fed evaporated milk, whole milk powder, and raw milk (unheated, autoclaved and frozen).

TABLE 3

Average nutritional values of milk protein

PROTEIN SOURCES	DIGESTIBILITY [1]	BIOLOGICAL VALUE [2]	NUTRITIVE INDEX [3]
	%	%	%
Raw milk (local)	98.6	100.8	99.3
Evaporated milk	109.2	103.6	113.1
Milk powder	102.3	103.7	106.1
Autoclaved raw milk — local (10 lb. for 15 minutes)	104.3	95.4	99.4
Autoclaved raw milk — local (10 lb. for 30 minutes)	103.4	97.2	100.3
Autoclaved raw milk — local (15 lb. for 30 minutes)	101.2	103.1	104.0
Frozen raw milk	104.9	107.8	112.8

[1] Digestibility (per cent) $= \frac{\text{Food N absorbed}}{\text{Total food N}} \times 100.$

Food N absorbed = Total food N — (Experimental fecal N — Control fecal N).

[2] Biological value (per cent) $= \frac{\text{Food N absorbed} - \left(\text{Experimental Urinary N} - \text{Control Urinary N}\right)}{\text{Food N absorbed}} \times 100.$

[3] Nutritive index = biological value × digestibility.

of 100%, assuming that the protein in the control milk is completely absorbed and utilized. Therefore, the digestibility and biological values of the experimental milk represent values relative to those of the control milk. A third quantitative expression of the nutritional efficiency of these milks has been obtained by multiplying the biological value by the digestibility, and it was termed by Cahill, Schroeder and Smith ('44) the "nutritive index" (see footnote, table 3).

The calculated values for digestibility for all the different types of milk used appear in figure 2. It is apparent that the

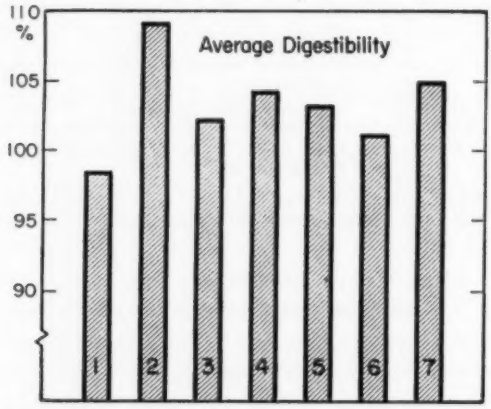

Fig. 2 Average digestibility of milk proteins in dogs fed evaporated milk, whole milk powder, and raw milk (unheated, autoclaved and frozen).

absorption of the protein of the autoclaved milk and milk products is almost the same as that of the control raw, unpasteurized milk. The digestibility of evaporated milk is slightly higher and this increase appears to be significant. Presented in another manner in figure 3, the total nitrogen output in feces revealed no appreciable increase in fecal nitrogen when the diet contained processed autoclaved milks, thus indicating a high degree of digestibility for these milks.

The average biological values can be seen in figure 4. From these data it is apparent that the absorbed nitrogen from the

experimental milks was retained and utilized as well as, or better than, that from the local raw milk. There was a slight, though not significant, decrease in nitrogen absorbed from the milk processed in our own laboratory. From figure 5, which shows the "nutritive indices" of the various types of milk, it can be seen more clearly that a slight difference existed

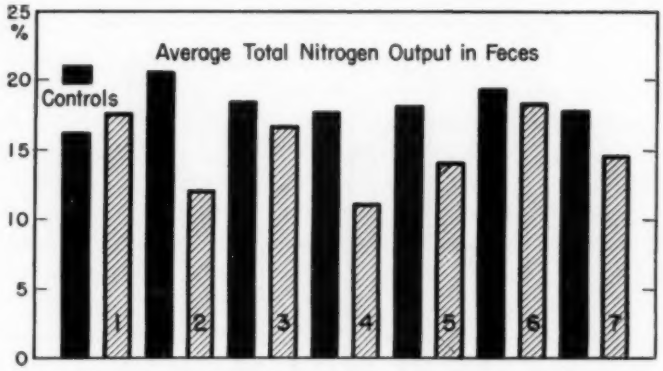

Fig. 3 Average total nitrogen output in feces of dogs fed evaporated milk, whole milk powder, and raw milk (unheated, autoclaved and frozen).

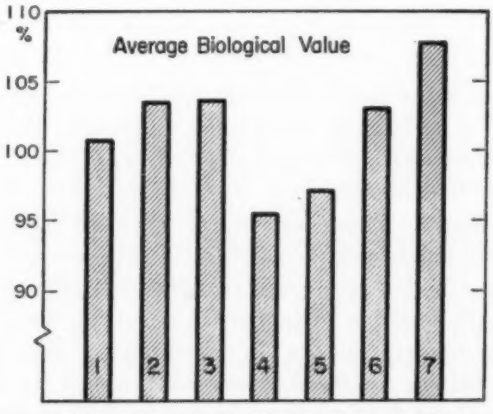

Fig. 4 Average biological value of milk proteins in dogs fed evaporated milk, whole milk powder, and raw milk (unheated, autoclaved and frozen).

among the various types of milk tested. Evaporated milk, due to its high digestibility, and milk kept in frozen storage, due to an inexplicably high biological value, appeared to have somewhat enhanced nutritive properties. A complete summary of digestibility, biological value, and the nutritive indices of the various milks studied appears in table 3.

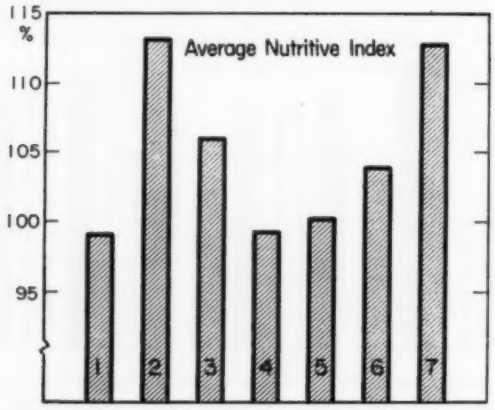

Fig. 5 Average nutritive indices of milk protein in dogs fed evaporated milk, whole milk powder, and raw milk (unheated, autoclaved and frozen).

DISCUSSION

It is clear from the results obtained in this investigation, under the experimental conditions employed, that heat as used in the commercial preparation of evaporated and dried whole milk does not decrease the nutritive value of the protein constituents. This confirms the work of Whitnah ('43), who noted in balance experiments with rats conducted according to the method of Mitchell ('24) that milk with high-quality protein could be either evaporated, canned or sterilized without loss of protein quality as measured by digestibility or biological value. Again, Hodson ('52), using the rat repletion method and feeding three levels (0.12, 0.18 and 0.24 gm per rat per diem) of protein nitrogen, failed to demonstrate

any difference in nutritive value between the protein of evaporated milk before sterilization and that after sterilization.

On the other hand, Cook, Morgan, Weast and Parker ('51) reported data on two different experiments, based on rat growth, which showed that the nutritive value of the proteins of two commercially prepared, evaporated milks was slightly but significantly lower than that of whole fresh pasteurized milk. In a later report, Kraft and Morgan ('51), comparing the nutritive value of unheated and autoclaved skim milk in both the rat and the dog, came to the conclusion that, while in the dog no striking changes in growth, appetite, nitrogen retention or serum protein could be demonstrated, in weanling rats the milk product autoclaved for 15 minutes lost from one-half to two-thirds of its growth efficiency.

Recently, Baldwin, Lowry and Thiessen ('51) reported that when mixtures of equal parts of protein (casein, $N \times 6.38$) and dextrose and one-half part of H_2O are processed at 15 pounds' pressure (121°C.) for three minutes, the protein efficiency is reduced by about 25%. Similar processing for 15 minutes resulted in such severe damage that the rats lost weight. However, the rat assay method used by Baldwin and associates has been the subject of some criticism. The addition of 9 essential amino acids as they appear in a 10% level of dietary casein, in addition to those already present in the heated casein in the diet, might well have resulted in amino acid imbalances that could influence the requirement of the one amino acid under test. The level of dietary protein (total amino acids) is an important factor in evaluating individual amino acid requirements.

The conflicting claims regarding the deleterious effect of heat upon the nutritive value of the proteins of milk cannot be centered in the difference in species of the experimental animals used, as the foregoing evidence shows. Rather, the diversity of observations would seem to arise from variation both in the intensity and duration of heating. One important factor in the protection of protein against heat damage, as will be shown in a subsequent paper, is the amount of mois-

ture present in the protein-containing system when the heat is applied.

SUMMARY

Tests on adult dogs have shown that autoclaving whole raw milk at 10 or 15 pounds' pressure for 15 or 30 minutes has no detrimental effect on the protein as measured by digestibility, biological value or nutritive index.

It was also found that the digestibility and biological value of milk protein do not change if milk is kept in frozen storage for 5 months.

It was observed that, under the experimental conditions employed, commercial processing as used in the preparation of evaporated milk and milk powder does not decrease the nutritive value of the protein constituents.

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EFFECTS OF BASAL DIETS ON THE RESPONSE OF RATS TO CERTAIN DIETARY NON-IONIC SURFACE-ACTIVE AGENTS [1]

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Considerable interest attaches to the study of the effects of prolonged feeding of non-ionic surface-active agents, since some of these compounds are used to technical advantage, in fractional percentages, in processing important food items such as bread and ice cream. Krantz ('49) and his associates have made extensive animal feeding studies on three classes of such emulsifiers, commercially designated the Span, Tween and Myrj types (the first two being partial esters of various fatty acids and sorbitan or polyoxyethylene derivative of stearic acid). They found that the ingestion of these substances by rats at levels of 2 to 5% in a commercial feed over a period of two years did not affect the growth rates or mortality and did not induce any gross or microscopic pathological changes in the experimental animals. Jones and his associates ('48) reported similarly that daily oral administration to humans of polyoxyethylene sorbitan monooleate (Tween 80) in doses of up to 15 gm daily for a period of several months and 4.5 to 6 gm daily for about 4 years (Krantz et al., '51) did not result in any observable effect.

[1] This work was supported by a grant-in-aid from the Atlas Powder Company. Span, Tween and Myrj are Atlas trade marks.

[2] The authors express appreciation for the technical assistance of Mrs. Shanley Davis.

Recently, Schweigert and his associates ('50) reported that feeding weanling hamsters polyoxyethylene monostearates at 5 or 15% levels in a diet containing purified casein, sucrose, a small amount of cellu flour, and salts, produced a significantly lower rate of gain in comparison with that observed in animals fed the same concentration of lard. In addition, these investigators (Wang, McBride and Schweigert, '50) also observed marked changes in the intestinal tract and severe diarrhea. The mortality rate, data on organ weights, and gross pathology led these workers to conclude that the ingestion of these compounds under their experimental conditions was deleterious to the hamsters. Harris and his associates ('50) found that feeding the laurate analogues of these surface-active agents to rats caused diarrhea, retardation of growth, and a higher mortality rate when they were incorporated at a 25% level into a diet similar to that used by Schweigert et al. ('50).

In view of these contradictory indications, we wish to present in this communication the data of our experiments in which both young weanling and year-old rats were given casein or sybean meal basal diets containing approximately 5 and 15% levels of three commercially available emulsifiers of the aforementioned type: Span 60 (sorbitan monostearate), Tween 60 (polyoxyethylene [20] sorbitan monostearate) and Myrj 52 (polyoxyethylene [40] monostearate).[3]

EXPERIMENTAL PROCEDURE

Animals used: Twenty-three-day-old weanling rats, of both sexes, weighing 45 to 55 gm for males and 40 to 50 gm for females were used in all but one experiment, where animals 12 months of age or older, weighing 300 to 350 gm for males and 200 to 260 gm for females, were employed. During the period of the studies, the rats were kept in individual cages and were weighed on every 7th day. Food intake data, if kept, were recorded weekly.

[3] See footnote 1, page 563.

Diets used: The compositions of the two basal diets as well as the experimental diets are: (a) basal soybean diet (BSD): soybean meal 62%, sucrose 29.5%, corn oil 4.5%, salt mixture IV 4.0%; (b) basal casein diet (BCD): casein 22%, sucrose 69.5%, corn oil 4.5%, salt mixture IV 4.0%; (c) test diets: into the basal soybean meal or casein diets were incorporated the desired levels of test emulsifiers. For example, "5% Tween 60 in basal soybean meal" consisted of 5.0 gm of Tween 60 plus 100 gm of the basal diet. "5% Tween in basal soybean meal + 8% cellu flour" consisted of 100 gm of 5% Tween 60 in basal soybean meal + 8 gm cellu flour. All diets were supplemented with water-soluble and fat-soluble vitamins in the following amounts per kilogram diet: 2.0 mg thiamine hydrochloride, 3.0 mg riboflavin, 2.5 mg pyridoxine, 20.0 mg Ca pantothenate, 50.0 mg niacin, 100.0 mg inositol, 0.10 mg biotin, 25.0 para-aminobenzoic acid, 0.0 mg folic acid, 1,000.0 mg choline HCl, vitamin A-1 250 I.U., vitamin D 180 I.U., menadione 2.1 mg, α-tocopherol 23 mg.

RESULTS

The effect of supplementation of the emulsifiers to the two basal diets on the growth rates of weanling rats:

At the 5% level: In experiment 1, female or male rats (23 days old) in groups of 12 each were fed ad libitum the following diets: A = basal casein diet (BCD), B = BCD + 5% Tween 60, C = basal soybean meal diet (BSD), D = BSD + 5% Tween 60, E = BSD + 5% Span 60, F = BSD + 5% Myrj 52, and G = BSD to which 100 µg of vitamin B_{12} were added to each kilogram of the mixture. The results of this experiment, given in table 1, indicate that the addition of Tween 60 to the basal casein diet (diets A and B) resulted in a marked retardation of growth of both male and female rats. Thus, after 8 weeks of feeding, for example, the male animals fed Tween 60 gained approximately one-half as much weight as the controls, while the female rats gained one-quarter less than the controls. Furthermore, all animals of-

fered diet B containing Tween 60 developed diarrhea [4] about 7 to 10 days after feeding. However, the severity of diarrhea decreased as feeding was continued. A comparison of the weight gains of both male and female rats in groups C, D, and E demonstrates that the supplementation of Tween 60

TABLE 1

Mean weight gains in grams of young weanling rats fed basal casein or soybean meal diets supplemented with non-ionic emulsifiers

GROUP [1]	EXPERIMENT 1 Weeks after feeding					EXPERIMENT 2 Weeks after feeding						
	2	4	6	8	10	2	4	6	8	10	12	14
	Males					Males						
A	40	108	168	228	...	40	104	159	224	255	288	296
B	28	67	99	108	...	38	63	109	166	206	226	232
C	59	108	159	191	224	66	131	186	224	247	259	270
D	56	103	146	179	205	59	133	187	216	229	246	269
E	71	129	154	196	214	56	120	166	208	233	258	268
F	..	...	...	...	...	69	126	184	216	236	243	266
G	68	130	168	228	244	68	145	200	246	266	286	291
	Females					Females						
A	55	88	118	146	...	44	88	117	139	151	164	174
B	32	57	78	112	...	26	60	98	126	140	155	146
C	55	86	106	129	144	47	79	99	120	133	150	162
D	52	81	109	125	144	48	89	118	133	145	150	149
E	52	83	109	131	140	41	90	114	115	125	141	153
F	..	..	...	...	...	56	93	117	137	142	155	172
G	55	87	120	136	144	51	84	110	125	134	135	151

BCD = basal casein diet.
BSD = basal soybean meal diet.
[1] A = BCD.
B = BCD + 5% Tween 60.
C = BSD.
D = BSD + 5% Tween 60.
E = BSD + 5% Span 60.
F = BSD + 5% Myrj 52.
G = BSD + vitamin B_{12}.

[4] The word "diarrhea" is used to describe the soft or molten conditions of the stool, although no morbidly profuse discharge from the intestine was observed.

or Span 60 to the basal soybean meal diet caused only a very slight retardation, if any, of growth rate among the male rats and none among the females, after 10 weeks of feeding. On no occasion was diarrhea observed in the male or female animals of these three groups. Since our soybean diet is deficient in vitamin B_{12}, it is not surprising to note that the male but not the female animals (group C) receiving the supplement of crystalline vitamin B_{12} gained the most weight.

Experiment 2 was a similar study which included an additional emulsifier, Myrj 52, and in which the period of feeding was 14 weeks. The number of male or female rats used for each group remained the same; namely, 12. In confirmation of the results of experiment 1, it was found in the first place that both male and female rats in group B receiving 5% Tween 60 in the basal casein diet did not gain as much weight as those in group A, particularly during the first 6 to 8 weeks of feeding, but the growth retardation effect seemed less distinct as feeding progressed to the 10th or 12th weeks. This improvement appeared to occur at a time when diarrhea, which developed initially, became less severe. In the second place, it can be pointed out that the animals in groups D, E, and F grew at essentially identical rates; no deleterious effects due to supplementation of BSD with the emulsifiers were observed. The deficiency of vitamin B_{12} in our diets containing soybean meal was again demonstrated by the greater weight gains of the male rats in group G, compared with the gains of the males in group C.

At the 15% level: In addition to the two experiments described above, the results of several other experiments of a similar nature were uniformly consistent, in that diarrhea and growth retardation regularly occurred, particularly during the early period of supplementation, with the emulsifiers at a 5% level in BCD but not in BSD. It was, therefore, of interest to ascertain whether supplementation with a higher level (15% of the emulsifiers in BSD) would be without any deleterious effects on weight gain. To this end, another experi-

ment with 23-day-old rats of both sexes was undertaken, with 12 animals, male or female, in each group. The compositions of the diets were the same as those used in experiments 1 and 2 except that in diets D, E, and F, 15 gm instead of 5 gm

TABLE 2

Mean weight gains in grams of young weanling rats fed basal casein or soybean meal diets supplemented with non-ionic emulsifiers

GROUP [1]	WEEKS AFTER FEEDING								
	1	2	4	6	8	10	12	14	16
					Males				
A	25	62	131	197	245				
B	29	62	122	172	192	187	220	251	245
C	24	55	104	147	183	222	258	272	274
D	30	64	109	161	190	227	260	277	294
E	10	30	66	96	134	165	184	201	223
F	19	53	102	145	176	208	240	273	292
					Females				
A	21	46	91	123	147				
B	20	50	87	113	127	132	143	146	154
C	20	44	78	105	125	138	146	149	154
D	24	43	86	111	131	168	163	157	162
E	23	16	56	81	101	120	128	129	130
F	26	46	81	116	135	154	153	159	182

BCD = basal casein diet.
BSD = basal soybean meal diet.
[1] A = BCD.
B = BCD + 5% Tween 60.
C = BSD.
D = BSD + 15% Tween 60.
E = BSD + 15% Span 60.
F = BSD + 15% Myrj 52.
G = BSD + vitamin B_{12}.

of Tween 60, Span 60 and Myrj 52 were added respectively to each 100 gm of the basal soybean diet. Supplementation of BCD with Tween 60 was repeated at the 5% level, since, in our experience, the addition of any one of the three non-ionic surface-active agents to BCD at this level uniformly

resulted in diarrhea and slower weight gain. These effects became more marked when the concentration of the emulsifiers was increased.

The weight gain data (see table 2) corroborate the earlier findings as to the effect of Tween 60 when incorporated in BCD (compare groups A and B). There was no significant difference in the increase of body weight among the groups of both male and female animals receiving BSD with or without supplementation by 15% Tween 60 (diet D) or 15% Myrj 52 (diet F). On the other hand, rats of either sex offered a diet supplemented with Span 60 (diet E) grew much less rapidly than the control animals (group C), even though none of the animals given this high level of emulsifiers developed any diarrhea. It was, therefore, of interest to ascertain whether the growth retardation due to Span 60 was related to a decrease of food or caloric intake. Food intake data for all groups of animals for the first 10 weeks of the experiment are shown in table 3. It can be seen, in the first place, that animals in group B consumed 10 to 15% less food or calories than their controls (group A) over the 10-week period during which food consumption records were kept. It is, therefore, conceivable that part, at least, of the growth retarding effect of Tween 60 in BCD during the initial period of feeding might well have been due to a smaller food or caloric intake. A comparison of the food consumption records among the remaining groups of animals demonstrates that only the animals in group E consistently consumed less food than those on other diets. It is, therefore, quite conceivable that the poorer growth of the animals on the Span diet is a reflection of smaller food or caloric intake. The difference in the caloric intake (see last column, table 3) becomes more accentuated when one recognizes that only a fraction of the fatty acid and none of the polyhydric portion of the molecule is actually utilized (Carr et al., '47; Culver et al., '51).

To demonstrate that the slower growth rate of young rats offered a diet containing Span 60 is related to the caloric intake, 23-day-old male or female rats in groups of 10 each

were fed the following diets: group A, BSD, ad libitum; group B, BSD + 15% Span 60, ad libitum; group C, BSD alone, fed with group B on total weight basis; group D, BSD + 15% Span 60, force-fed to achieve greater intake than group B. The records of the increase in body weight and food consumption are given in table 4.

TABLE 3

Mean cumulative food intakes of young weanling rats fed basal casein or soybean meal diets supplemented with non-ionic emulsifiers

(Same animals as in table 2)

GROUP	FOOD CONSUMED (IN GRAMS) AFTER WEEKS OF FEEDING						MAX. CALORIC VALUE [1]	TOTAL CALORIC INTAKE AFTER 10 WKS.
	1	2	4	6	8	10		
				Males				
A	45	110	220	410	596	788	4.1	3,230
B	43	104	184	366	539	673	4.0	2,690
C	45	99	243	444	648	867	3.4	2,950
D	49	115	269	477	678	898	3.3	2,960
E	39	72	196	360	516	704	3.8	2,680
F	42	104	256	462	666	889	3.1	2,670
				Females				
A	40	100	240	396	528	672	4.1	2,760
B	39	91	220	362	495	605	4.0	2,420
C	39	95	231	383	529	664	3.4	2,260
D	45	104	256	420	588	752	3.3	2,480
E	26	59	199	325	468	617	3.8	2,340
F	44	100	252	439	621	812	3.1	2,520

[1] Calculated on the basis that the fatty acid in the emulsifier molecule is completely utilized.

The results show that the food intakes of rats in groups A, B, and C were essentially identical over the 10-week period. Under these circumstances, animals in groups A and C should have gained weight at the same rate. Actually, the growth rate in the latter group was slightly poorer. Animals in group B did not grow as rapidly as litter mates in group C. Although both groups of animals consumed the same amount of food, rats in group B received fewer available calories be-

cause of the fraction of surface-active compound which is not completely metabolized. When the food intake was increased by daily forced feeding (group D), the growth rate was raised and became equal to that for the animals in group A. We attribute this improvement to a greater intake of available

TABLE 4

The effect of food consumption on the growth rate of weanling rats offered BSD with or without supplementation with Span 60

DIET [1]		WEEKS AFTER FEEDING									
		2	4	6	8	10	2	4	6	8	10
		Males					Females				
A	F.C.[2]	102	262	446	650	830	95	240	399	567	720
	Wt. gain	42	97	142	181	199	35	85	108	133	140
	F.C./Wt. gain	2.4	2.7	3.1	3.6	4.2	2.7	2.8	3.7	4.3	5.1
B	F.C.	102	249	429	633	819	87	232	393	578	734
	Wt. gain	33	75	107	150	167	28	71	96	116	126
	F.C./Wt. gain	3.1	3.3	4.0	4.2	4.9	3.1	3.3	4.1	5.0	5.8
C	F.C.	99	251	427	622	610	94	238	391	559	708
	Wt. gain	40	93	137	173	197	33	83	103	127	135
	F.C./Wt. gain	12.5	2.6	3.1	3.6	4.1	2.8	2.9	3.8	4.4	5.2
D	F.C.	112	272	478	...	...	98	252	436	...	...
	Wt. gain	44	100	141	...	...	34	79	105	...	...
	F.C./Wt. gain	2.5	2.7	3.4	...	...	2.9	3.2	4.2	...	...

[1] Diet A = Basal soybean meal diet BSD fed ad libitum.
Diet B = BSD + 15% Span 60, fed ad libitum.
Diet C = BSD restricted to the intake of group B.
Diet D = BSD + 15% Span 60 with forced feeding.
[2] F.C. = Food consumed, in grams.

calories. This fact is not in agreement with the hypothesis that the growth retardation observed following incorporation of Span 60 into the ration (groups B and C) was due to any toxicity of the emulsifier. If this toxicity hypothesis is valid, the animals in group D should have grown at a less favorable rate, since they ingested more Span 60 than the animals in group C; the data are opposed to this expectation.

The effect of supplementation of BSD with Tween 60 on the body weights of old rats: In view of the fact that old rats may be more susceptible to diarrhea and loss of body weight than adult rats, male and female animals older than one year were used for this study. They were kept in individual cages for approximately 4 weeks, during which time they were fed BSD and weighed once a week. Under these conditions their body weights were found not to vary by more than $\pm$ 10 gm. These animals were then divided, first into two groups according to their sexes and then into three subgroups of 12 each by random distribution. The average weights of the animals in each

TABLE 5

Mean changes (in grams) of body weight in old rats[1]

WEEKS OF FEEDING	DIET A		DIET B		DIET C	
	Male	Female	Male	Female	Male	Female
1	3	−5	9.2	1	10	1
3	−4	−6	−15	−15	14	4
5	5	0	12	4	56	8
7	2	−6	12	−2	43	5
9	10	20	21	1	48	7
11	23	2	30	−6	58	10
13	36	−7	24	−2	63	4

[1] Minus meaning loss of weight. Others gained weight.

group were, for males, 340 $\pm$ 10 gm; for females, 230 $\pm$ 7 gm. The diets offered to these three groups were: A = basal soybean meal diet (BSD); B = BSD + 5% Tween; and C = BSD + crystalline vitamin B_{12} (100 µg per kilogram diet). The changes in body weight as a function of time are given in table 5. They demonstrate that the body weights of the animals on BSD fluctuated only slightly during the first 9 weeks; definite changes are seen with the males thereafter. The males in group B gained about 24 gm in 13 weeks, while the females in the same group, like those in the control group, did not appear to gain or lose weight. Supplementation of BSD with crystalline vitamin B_{12} stimulated the weight gain of animals in group C, especially the males.

In the experiment just described, the stools of the animals receiving Tween 60 appeared to have the same consistency as those of rats on control or vitamin B_{12}-supplemented diets. On the other hand, when rats of the same age were fed 5% Tween 60 added to BCD, diarrhea developed in about 10 days and continued for three to 5 weeks. The average body weight of these animals was less in the 5th week by 25 gm, and in the 10th week by 2 gm, than that of their controls fed BCD.

From these data it is apparent that old animals, like the young weanlings, did not develop diarrhea or lose weight when Tween 60 was added to BSD at a 5% level, but they did develop diarrhea with concomitant loss of body weight when this emulsifier was added to BCD. However, as the experiment continued, diarrhea stopped and the animals regained most of the lost weight.

DISCUSSION

Since non-ionic emulsifiers are now used to technical advantage in the processing of widely used common foods, objective studies which can evaluate the effects of their administration under controlled conditions are of paramount importance. Some investigators have reported observing diarrhea as a result of the feeding of certain non-ionic emulsifiers to experimental animals. According to their reports, this symptom was usually followed or accompanied by poor food consumption, growth retardation or even increase in mortality. These effects have been attributed to the intrinsic toxic characteristics of the surface-active agents. Our findings illumine these results from another perspective, and provide bases for more realistic assessment of the effects of feeding these emulsifiers.

The results of the experiments described above demonstrate that feeding any one of the three emulsifiers at levels of 5% or 15% of a basal soybean meal diet, to young weanling or old rats of both sexes, does not result in diarrhea or growth retardation, although these effects are manifest

when an emulsifier, exemplified by Tween 60, was added to a basal casein diet. Although the latter is generally considered to be adequate in essential nutrients, it does not contain much of the unabsorbable substances which, when present, can form a considerable part of the stool. Hence, animals on such a completely digestible diet have much smaller fecal matter bulk than those on a diet containing soybean meal. This lack of adequate bulk to mix with unabsorbed emulsifier permits the emulsifier to cause some physiological changes due to its surface-active properties. Histological examination of sections of kidney, testis, liver, spleen, pancreas, and intestines (jejunum and sigmoid) taken from male animals fed these basal diets with 5% and 15% added emulsifiers failed to reveal any significant abnormalities. There was no evidence of damage to the bowel or liver, as suggested by Wang and associates ('50), although it must be pointed out that their findings were made with weanling hamsters instead of rats. Furthermore, these investigators used nonionic surface-active agents whose chemical structures differ from those reported in this communication in the hydrophylic portion of the molecule. Confirming our histological evidence for an essentially undamaged intestinal mucosa was the fact that the benzidine test for occult blood was negative when applied to the stools from these animals. Fat stains (Sudan IV) of the kidneys and livers from the same animals likewise showed no abnormal fat accumulation.

It is possible to offer other explanations for our results, such as the presence of some substance in soybean meal which might counteract these effects of the emulsifying agents. Since there is no evidence of the existence of such a substance, we prefer to postulate that the development of diarrhea under some conditions is due, primarily, to lack of residues in the basal diet to absorb the surface-active agent, which can be irritating to the intestinal tract by virtue of its physical properties. This hypothesis receives further support in the results of a series of experiments which demonstrated that the addition of bulk-forming inert substances such as cellu

flour (8%), or celite (6%), or agar (8%), to the casein diet prevents diarrhea.

To ascertain whether prevention of diarrhea will also result in the correction of growth retardation, 23-day-old weanling rats of both sexes were fed the following diets:

A = basal casein diet, BCD + 10% Myrj 52, ad libitum;
B = BCD + 10% Myrj 52 + 8% cellu flour, ad libitum and
C = BCD with protein intake restricted to that of B.

TABLE 6

The effect of cellu flour on the growth (in grams) of weanling rats fed a diet containing Myrj 52

DIET[1]		MALE			FEMALE		
		1 wk.	3 wk.	5 wk.	1 wk.	3 wk.	5 wk.
A	F.C.[2]	39	168	332	37	167	324
	Wt. gain	11	56	107	10	54	94
	F.C./Wt. gain	3.5	3.0	3.1	3.7	3.1	3.4
B	F.C.	44	186	345	33	170	318
	Wt. gain	14	63	119	6	58	89
	F.C./Wt. gain	3.1	3.0	2.9	5.5	2.9	3.6
C	F.C.	37	140	299	35	140	302
	Wt. gain	15	64	122	12	61	93
	F.C./Wt. gain	2.5	2.2	2.5	2.9	2.3	3.2

[1] A = Basal casein diet (BCD) + 10% Myrj 52, fed ad libitum.
B = BCD + 10% Myrj 52 + 8% cellu flour, fed ad libitum.
C = BCD with protein intake restricted to that of group C.
[2] Food consumed, in grams.

The results demonstrate that animals (group A) ingesting 10% Myrj 52 did not grow as rapidly as those receiving both Myrj 52 and cellu flour, in spite of the fact that animals in the latter group consumed approximately the same amount of food of less caloric density. It is probable that the better utilization of food by animals in group B is associated with the prevention of diarrhea with cellu flour. As a matter of fact, in the absence of diarrhea the animals consuming both the emulsifier and cellu flour increased their body weight as rapidly as those (group C) on casein alone, pro-

vided the protein and caloric intakes were essentially the same.

SUMMARY

When 23-day-old weanling rats were fed a basal casein diet supplemented with 5% polyoxyethylene sorbitan monostearate (Tween 60), diarrhea developed during the initial period of feeding and the growth rate was retarded. On the other hand, supplementation of this surface-active substance or of two similar agents, such as Myrj 52 or Span 60, to a soybean meal basal diet, even at a 15% level, did not produce these deleterious effects except in the animals on Span 60. These rats consumed less food and consequently grew at a slower rate than those on other emulsifiers or those serving as controls. At the end of 14 weeks of feeding, animals on both 15 and 5% levels were sacrificed. No abnormal histological or pathological changes were observed. To test whether older rats would be more susceptible to any possible deleterious properties of the agents, rats of one year of age or older were fed 5% Tween 60 in the soybean basal diet for 14 weeks. No loss of weight or development of diarrhea was observed. From these results it was concluded that, depending on the basal diet used for supplementation, each of the three nonionic emulsifiers investigated in this study produced no observable toxic effect.

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FURTHER STUDIES RELATING LIVER XANTHINE OXIDASE TO QUALITY OF DIETARY PROTEIN [1]

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Recently a method has been described for estimation of protein quality using the response in liver xanthine oxidase activity as a criterion (Litwack et al., '52). We have found this response in liver xanthine oxidase activity generally to parallel growth in weanling rats fed the same protein. Earlier, Williams and Elvehjem ('49) observed an increased availability of amino acids from casein after acid hydrolysis, using xanthine oxidase activity as the criterion. Later Litwack et al. ('50) found that the rapid loss of enzyme activity, when animals are fed a non-protein diet, is due primarily to a loss of the enzyme protein rather than to a deficiency of cofactors required in the enzyme system. The rapidity of *in vitro* xanthine oxidase formation was recently demonstrated by Dhungat and Sreenivasan ('52).

The activity of this enzyme has also been shown to be maintained by nitrogen sources, other than amino acids, which effectively replaced a non-essential amino acid mixture which was added to a basal level of the essential amino acids (Lit-

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[2] Fellow of the Williams-Waterman Fund for the Combat of Dietary Diseases.

wack, Williams and Elvehjem, '53). This evidence, together with experiments which show the lability of xanthine oxidase to certain essential amino acid deficiencies (Williams, Denton and Elvehjem, '49; Williams and Elvehjem, '49), suggested that the enzyme activity was related to protein quality under certain conditions (Litwack et al., '52) and that this "protein quality" might truly represent the value of a protein with respect to its efficiency in promoting synthesis of protein in the tissues of the body. This latter possibility has been investigated and the results are presented in this paper, with special reference to the effects obtained by supplementing a dietary protein deficient in certain amino acids with those amino acids known to be lacking The responses in liver xanthine oxidase activity could then be compared to the response given by the complete protein, casein, which was used as the standard.

EXPERIMENTAL

In the enzyme experiments, adult male albino rats [3] were allowed a 7-day adjustment period on a good stock ration before being placed on the experimental diets. Weanling rats of the same strain weighing between 45 and 50 gm were employed in the growth experiments and received a similar adjustment period.

The basal diets consisted of the following: protein, 5, 10, 18 and 30 gm; vitamin mix (Williams and Elvehjem, '49), 2 gm; salts IV (Hegsted et al., '41), 4 gm; corn oil, 5 gm and sucrose to make 100 gm. Any additions to this diet were compensated for by reducing the level of sucrose. The animals were housed individually in screen-bottomed cages and received food and water ad libitum. The fat-soluble vitamins were administered weekly in the form of two drops of halibut liver oil given orally. Weight data were also recorded each week. A total of 173 animals were used in the enzyme studies and 60 weanling animals were employed for the growth experiments.

[3] Obtained from the Holtzman-Rolfsmeyer Rat Co., Madison, Wisconsin.

Hydrolyzed gliadin was prepared by autoclaving purified gliadin [4] at 15 pounds' pressure for 12 hours with 10 volumes of 3 *N* sulfuric acid and neutralizing the hydrolysate with powdered calcium hydroxide. The calcium sulfate precipitate was washed with steam and the washings were combined with the filtrate. The filtrate was concentrated by steam vacuum distillation at 50° to a thick syrup. This was dried in a vacuum oven at 55° and the product was ground to a fine powder in a Wiley mill. The amino acid content of the fine homogeneous powder was then analyzed microbiologically for the essential amino acids, using a modification of the medium of Henderson and Snell ('48). Since destruction of some of the amino acids occurred during the hydrolysis procedure, the hydrolysate was reconstituted by addition of amino acids to the levels for whole gliadin as indicated by Block and Bolling ('51).

Each protein or supplemented protein was fed at 4 levels, 5, 10, 18 and 30% of the ration. Casein was used as the standard preparation. In the experiments involving gliadin and various supplements, the rations were prepared isonitrogenously to the corresponding level in the casein standard as measured by Kjeldahl-determined nitrogen. When individual amino acids were added to whole gliadin, they were included at the following levels: L-lysine·HCl, 6.2% and DL-tryptophan, 1.0% of the protein preparation. When acid-hydrolyzed gliadin was reconstituted with amino acids to the levels found in whole gliadin, the following levels of amino acids were added: L-arginine·HCl, 0.16%; L-lysine·HCl, 0.19%; DL-tryptophan, 0.77%; DL-methionine, 0.78%; and DL-threonine, 0.57% of the protein preparation. When whole gliadin was brought to the amino acid content of casein, the following levels of amino acids were added: L-arginine·HCl, 1.5%; L-lysine·HCl, 6.2%; DL-tryptophan, 1.0%; DL-methionine, 1.4%; DL-threonine, 1.2%; DL-leucine, 4.86%; DL-isoleucine, 2.19%; DL-valine, 4.09% and DL-alanine, 3.2% of the protein preparation.

[4] Huron Mills.

The enzyme and growth experiments and calculations were carried out as previously indicated (Litwack et al., '52).

RESULTS AND DISCUSSION

The results of the enzyme experiments are presented in table 1. Before considering these results, a clarification of the calculation for the xanthine oxidase response should be

TABLE 1

Response of liver xanthine oxidase activity in rats to various dietary proteins

DIETARY PROTEIN	NO. OF RATS USED IN THE DETERMINATION	XANTHINE OXIDASE RESPONSE CONSTANT IN μL OF O_2 PER HR. PER % OF DIETARY PROTEIN	
		N [1] = 3	N [1] = 4
Casein	58	5.9 ± 0.6 [2]	5.0 ± 0.4 [2]
Gliadin	34	1.0	2.0
Reconstituted hydrolyzed gliadin	13	0.6	0.8
Gliadin + tryptophan	26	1.2	2.2
Gliadin + lysine	16	4.0	3.2
Gliadin + lysine + tryptophan	12	5.1	3.9
Gliadin + amino acids to simulate casein	14	4.2	3.9

[1] N = number of levels of dietary protein used in the calculation. For N = 3: 5, 10, and 18% levels of protein were employed. For N = 4: 5, 10, 18, and 30% levels of protein were employed.

[2] Standard error of the mean. This calculation is omitted from the other values, since most of the remaining groups have too few final values to present a meaningful standard error. However, the average standard error for all groups is approximately ± 10%.

made. The method for calculation of protein quality, as described in a previous communication (Litwack et al., '52), involved 4 observations at 5, 10, 18 and 30% levels of protein in the diet. It has since been noted that the enzyme activity for the 30% protein group invariably falls at a point where the experimental curve has levelled off or begins to level off. This factor, therefore, tends to lower the xanthine oxidase efficiency constant. For this reason two values for

the enzyme response constant have been reported in table 1: one in which the 4 protein levels have been used and the other where the observations for the first three protein levels were employed. The authors feel that the constants reported using the three protein levels are more valid than those calculated from the 4 values, since the levelling-off effect is negated by omitting the last (30% protein) value.

From the results in table 1, it can be seen that the highest xanthine oxidase response constant was obtained when casein was the test protein. A marked decrease in this value was found with unsupplemented whole gliadin. In order to observe whether the low response to whole gliadin was due in part to poor digestibility of the protein as well as its lack of lysine and tryptophan, gliadin was hydrolyzed and reconstituted to the level of amino acids present in whole gliadin as described above. Kligler and Krehl ('50, '52), using zein as a test protein, have indicated that supplementation of that protein with lysine did not support as good growth in rats as that obtained with casein. They concluded that zein is poorly digested by the rat and found that even in the presence of adequate amounts of lysine, growth was not comparable to that produced by casein. The results with acid-hydrolyzed gliadin, as shown in the table, indicate that the enzyme response to hydrolyzed gliadin was lower than the response to whole gliadin. Therefore it appears that increasing the availability of the amino acids in gliadin by hydrolysis actually depressed its "protein quality." If the amino acid ratios in gliadin were already conducive to an amino acid imbalance, this effect would probably be amplified by feeding the protein in hydrolyzed form. Kligler and Krehl ('50) partially explained their results with zein in terms of this concept of amino acid imbalance.

When tryptophan was added to the whole gliadin to bring it to the level present in casein, a response constant resulted which was not greater than that for the whole gliadin alone. On the basis of the essential amino acid content of gliadin, one could not expect the addition of tryptophan to overcome

both a lysine and a tryptophan deficiency. However, when gliadin was supplemented with lysine alone to the level found in casein, a marked increase in the response constant was observed. This indicated that tryptophan was much less limiting as far as the xanthine oxidase response was concerned than lysine under these conditions. One would, however, expect to enhance the value of the protein greatly by the addition of both lysine and tryptophan and, indeed, this appears to be the case. The addition of both lysine and tryptophan gave an added response, though the constant was still not as high as that obtained with casein.

In the final enzyme experiments, all of the essential amino acids in whole gliadin were brought to the same levels as those in casein. The resulting enzyme response constants were no higher than the values obtained when tryptophan and lysine were the only supplements. The results indicate further that the relative concentrations of amino acids in whole gliadin are such that even when the lacking essential amino acids are brought to the levels found in casein, the resulting protein still is not as good as casein. A similar effect has been noted by Kligler and Krehl ('50) as well as other workers (Holt and Albanese, '44; Geiger and Hagerty, '49). It is possible, since vitamin B_{12} was not included in the rations, that this might account for the greater effect of casein than of gliadin supplemented with amino acids. However, since adult rats, with presumably adequate stores of vitamin B_{12}, were employed in these experiments, it is doubtful that a vitamin B_{12} deficiency would be induced during the 10 days of feeding the various rations.

In order to investigate the correlation of the xanthine oxidase response with the growth-promoting ability of proteins, the growth of weanling rats fed several of the dietary proteins listed above was studied. The results for these experiments were calculated in the same manner as the xanthine oxidase data. The results of these experiments, which are reported in table 2, indicate that casein promoted growth response to the greatest extent. A markedly lowered growth

response constant was obtained with gliadin, as was the case in the enzyme experiments. When tryptophan was added to gliadin, no increase over the response constant for gliadin alone was noted. Since tryptophan alone supported no better growth than gliadin alone, this again indicated the inability of tryptophan to exert an effect while lysine was still deficient. The addition of lysine alone corrected the deficient status of the protein, as measured by the growth method, which was also demonstrated in the enzyme experiments. Lysine appears, then, to be the most important factor lacking in gliadin to stimulate the processes of growth as well as enzyme

TABLE 2

Response of weanling rats in growth to some of the dietary proteins studied

DIETARY PROTEIN	NO. OF RATS USED IN THE DETERMINATION	GROWTH RESPONSE CONSTANT IN GRAMS OF GAIN PER RAT PER WEEK PER % OF DIETARY PROTEIN	
		$N^1 = 3$	$N^1 = 4$
Casein	12	2.1	1.3
Gliadin	12	0.49	0.47
Gliadin + tryptophan	12	0.49	0.52
Gliadin + lysine	12	2.0	2.7
Gliadin + lysine + tryptophan	12	1.8	1.5

[1] See footnote 1 in table 1.

formation. When both tryptophan and lysine were present in the diet with gliadin, the growth response constant was approximately the same as for protein supplemented with lysine alone, again indicating that the deficiency of lysine in gliadin is of far more importance to the rat than the deficiency of tryptophan.

A final evaluation for the calculation of the enzyme constant based on the observations from three protein levels (5, 10 and 18%) is shown in table 3. Here the ratios between the xanthine oxidase response constant and the growth response constant for corresponding experiments are listed. In each case, calculation of these constants using the three and

4 observations may be compared. It can be seen that in nearly all cases, a much more constant ratio between the enzyme and growth method is obtained if the results from three observations (at 5, 10 and 18% protein) rather than 4 levels (including 30% protein) are employed in the calculation.

It seems likely, in view of the correlation of the enzyme results with the growth results, that the enzymatic method measures the value of a protein with regard to the status of metabolism and tissue synthesis in the animal receiving the protein in much the same manner that growth does.

TABLE 3

Ratio of the xanthine oxidase response constant to the corresponding growth response constant for various dietary proteins

DIETARY PROTEIN	XANTHINE OXIDASE RESPONSE CONSTANT/ CORRESPONDING GROWTH RESPONSE CONSTANT	
	$N^1 = 3$	$N^1 = 4$
Casein	2.8	3.8
Gliadin	2.1	4.3
Gliadin + trytophan	2.4	4.2
Gliadin + lysine	2.0	1.2
Gliadin + lysine + tryptophan	2.8	2.6

[1] See footnote 1 in table 1.

In 1919, Osborne, Mendel and Ferry introduced the con cept of "protein efficiency ratio" as a refinement of the simple growth method. In general they found that the better the protein, the lower the level in the diet required to produce the highest efficiency ratio. Other more specific criteria exist, and it is likely that few ultimate evaluations can be made until all of these criteria are understood and synthesized to show effects on the whole animal. We are interested, primarily, however, in the stimulation of protein synthesis in the body by exogenous protein sources which may be used as a standard for protein quality. Since xanthine oxidase activity is a measurement of the formation of that enzyme protein, it should be a suitable criterion for estimation of pro-

tein value. Furthermore, the enzyme method produces results in general agreement with the effects upon growth and seems to obviate some of the interferences encountered in the growth method, such as the hormonal state of the animal, water intake and water balance, and fat deposition.

SUMMARY

1. Studies with a deficient protein, gliadin, have indicated that the response in liver xanthine oxidase as a basis for measuring protein quality is very sensitive to changes in the state of the protein with respect to its amino acid composition.

2. Calculation of the enzyme response constant has been analyzed and revised to represent a refined value for protein quality.

3. Enzyme studies with casein, whole gliadin, and gliadin supplemented with various amino acids concur very well with similar studies where growth has been used as a criterion.

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THE INFLUENCE OF RELATIVE HUMIDITY UPON VITAMIN DEFICIENCIES IN RATS[1]

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ONE FIGURE

(Received for publication September 19, 1952)

INTRODUCTION

Although the influence of environmental temperature upon the nutrition of the laboratory rat has been studied by Mills ('41, '42, '43) and others, little information is available regarding the effects of relative humidity. For this reason the action of extremely high and extremely low relative humidity conditions upon weanling rats was studied. Since it is known that relative humidity influences the severity of scaliness in nutritional dermatoses, the vitamin deficiencies which produce dermatoses were used. A preliminary report by Collins, Schreiber, Niño-Herrera and Elvehjem ('52) has been presented.

EXPERIMENTAL

Weanling male rats of the Sprague-Dawley strain were fed a complete diet and diets deficient in vitamin A, riboflavin, pantothenic acid or pyridoxine. The composition of the complete diet is presented in table 1. To prepare each of the vitamin-deficient diets, the appropriate vitamin was omitted.

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The diets were fed ad libitum. Water was supplied to each animal from inverted water bottles.

The animals were housed in individual raised screen bottom cages and were maintained in chambers at controlled relative humidities of 5 to 10%, 50%, and 90% and in our animal room at 30%. A constant temperature of 26°C. was maintained throughout all experiments. The relative humidity and temperature within the chambers were determined by the use of hygrothermographs which were calibrated against a high-precision direct-reading hygrometer and a standard thermometer.

TABLE 1

Composition of complete diet

Cottonseed oil	5%		
Sucrose	73%		
Casein (vitamin-free)	18%		
Salts IV [1]	4%		
Vitamins (mg per 100 gm of diet)			
Thiamine	0.2	Folic acid	0.2
Riboflavin	0.3	Vitamin B_{12}	0.002
Pyridoxine	0.2	Choline chloride	100.0
Nicotinic acid	2.5	Carotene [2]	0.5
Ca pantothenate	2.0	Calciferol	0.005
Inositol	10.0	α-Tocopherol	3.0
Biotin	0.01	Menadione	1.0

[1] Hegsted et al. ('41).

[2] Ninety per cent beta and 10% alpha.

The low relative humidity of 5 to 10% was maintained by controlling the flow of dry air into the chamber and by absorbing urinary and fecal water with anhydrous $CaCl_2$. The dry air was prepared by bubbling compressed air through a series of bottles containing concentrated sulphuric acid. This air was then passed through glass tubes filled with cotton and soda lime to remove acid vapors. A second chamber was maintained at 50% relative humidity by passing in dry air without the use of a dehydrating agent within the chamber. The high relative humidity chamber was kept near 90%

by introducing moist air into the chamber and by placing trays of water-soaked wood shavings beneath the animals.

To maintain these different humidities, the required flow of air into the chambers was 36, 10, and 6 l per minute for the 5 to 10%, 50% and 90% relative humidity conditions, respectively. The estimated oxygen consumption for 12 100-gm rats at an oxygen uptake rate of twice basal conditions was calculated to be approximately 60 ml of oxygen per minute (Mitchell and Carman, '26). It is assumed that sufficient oxygen was being administered to the animals in all of the chambers, since at the lowest flow rate of 6 l of air per minute (90% relative humidity chamber), 1,200 ml of oxygen were entering each minute.

Because each chamber could only accommodate 12 animals, small groups of from two to 6 rats were used; however, the experiments were repeated from two to 4 times.

RESULTS AND DISCUSSION

Growth, efficiency of food utilization, and water consumption

As is shown in table 2, extreme humidity conditions were not found to influence the growth of rats fed the complete diet (group 1),[3] the vitamin A-deficient diet (group 2), or the pantothenic acid-deficient diet (group 4). The riboflavin-deficient rats grew more slowly at 5 to 10% relative humidity, but when these animals were supplemented with a low level of riboflavin, 10 μg per rat per day, growth was similar under all humidity conditions (groups 3 a and 3 b). The animals receiving the pyridoxine-deficient diet and the pyridoxine-low diet (groups 5 and 6) grew more rapidly at the 90% relative humidity condition than at lower relative humidities. The efficiency of food utilization also tended to be greater among these pyridoxine-deficient rats at the 90% relative humidity condition during the third and 4th weeks.

[3] The initial observation by Collins et al. ('52) that high humidity favored the growth of rats fed a complete diet does not appear to be consistent with this finding.

TABLE 2

The influence of relative humidity upon rat growth[1]

	DIET	TOTAL NUMBER OF RATS/ DIET	NUMBER OF RATS MAINTAINED AT EACH RELATIVE HUMIDITY	GROWTH PERIOD	CONTROLLED HUMIDITY CHAMBERS			RAT ROOM
					5–10%	50%	90%	30%
				weeks	*gm gain*	*gm gain*	*gm gain*	*gm gain*
1.	Complete	32 (3)[2]	8	6	213 ± 6.4[3]	202 ± 7.2	201 ± 4.6	190 ± 5.2
2.	— Vitamin A	16 (2)	4	3	42 ± 7.0	45 ± 1.6	45 ± 6.0	53 ± 2.5
3a.	— Riboflavin	16 (2)	4	3	4 ± 1.5	12 ± 1.6	10 ± 4.8	8 ± 1.1
3b.	+ 10 μg riboflavin/ rat/day	16 (2)	4	3	38 ± 4.7	38 ± 6.3	40 ± 7.2	36 ± 9.8
4.	— Pantothenic acid	32 (3)	8	3	36 ± 6.5	38 ± 9.1	39 ± 5.8	39 ± 7.0
5.	— Pyridoxine	56 (4)	14	4	27 ± 2.8	32 ± 1.7	44 ± 2.4	34 ± 1.1
6.	Low pyridoxine 0.3 mg/kg diet	24 (1)	6	6	77 ± 5.5	87 ± 5.0	97 ± 5.7	73 ± 6.3

[1] Temperature kept constant at 26°C.
[2] Figures in parentheses represent the number of experiments.
[3] Standard error of the mean.

Since water consumption has been shown to be a function of the body area (Richter and Brailey, '29), the average daily water consumption was calculated in terms of body surface. The water intake per unit of body area of rats fed a specific diet at a specific humidity was found to be relatively constant throughout the growth period; however, the consumption of water decreased as the relative humidity increased (table 3). Pyridoxine-deficient rats drank less water than rats receiving the pantothenic acid-deficient diet or the complete diet.

TABLE 3

Average daily water consumption per rat per square decimeter of body surface[1]

RELATIVE HUMIDITY	COMPLETE DIET	LOW PYRIDOXINE (0.3 mg/kg diet)	PYRIDOXINE-DEFICIENT	PANTOTHENIC ACID-DEFICIENT
	4 rats for 6 wks.	6 rats for 6 wks.	10 rats for 4 wks.	4 rats for 3 wks.
%	*ml*	*ml*	*ml*	*ml*
5–10	6.5	4.0	3.9	6.3
50	4.2	3.6	3.1	5.3
90	4.8	2.3	2.7	4.3

[1] Body surface area was calculated by the method of Carman and Mitchell ('26).

Dermatosis

At 5 to 10% relative humidity scaly dermatosis occurred on the paws of all rats, those receiving the complete diet as well as those receiving the vitamin-deficient diets. The dermatosis resulting from the complete diet was characterized by dehydration of the skin of the paws, cracking, and maximum scale formation by about 12 days; after this time the scales flaked off, leaving new smooth skin. This recovery process appeared very similar to the spontaneous recovery of weanling rats showing dermatosis resulting from lactose diets (Schreiber, Collins, Niño-Herrera and Elvehjem, '52). Following recovery, the skin of the paws did not develop further scaliness; however, some dehydration on the ventral side did occur. The inclusion of 0.05% iodinated casein in the complete diet decreased the severity of the dermatosis.

At 30% relative humidity, scaly dermatosis occurred only in the rats receiving vitamin-deficient diets and not in those receiving the complete diet, while at 50% and 90% relative humidity scales were not observed on the paws of any of the animals. Even though no scales formed on the paws of vitamin-deficient rats at relative humidities of 50% and above, histological changes in the skin tissue may have occurred. Niño-Herrera, Schreiber, Collins and Elvehjem ('53) have found this to be true in the case of the dermatosis resulting from lactose diets.

Red pigment excretion

During each of the 4 experiments conducted, the animals which were maintained at 90% relative humidity developed within a period of one to three weeks, about the head and body, a fur which was stained with a red pigment. This condition was nearly non-existent with animals maintained at relative humidities of 50% and below. The greatest extent and incidence of this phenomenon occurred among the rats receiving diets deficient in riboflavin, pantothenic acid, and pyridoxine and to a lesser degree among vitamin A-deficient rats, while rats receiving a complete diet were only very slightly affected. A moderate distribution of this red pigment is seen on the head of the pyridoxine-deficient rat in figure 1.

The red pigment appeared first upon the tips of the hair about the forward part of the head, and is believed to have arisen from the nostrils. The pigment may be spread by the animal's fore-paws to the tips of the fur of the head and, in some cases, transferred from the head to the back and flanks of the rat by direct contact.

The chemical nature of this material has not yet been investigated; however, it gave a red fluorescence when illuminated with ultraviolet light similar to that of hemoglobin and that of the porphyrin pigments arising from the Harderian glands as a result of vitamin A deficiency or acetylcholine-induced chromodacryorrhea.

The occurrence of red-stained fur on vitamin-deficient rats has been observed by many investigators. Lemberg and Legge ('49) have reviewed the porphyrin metabolism of the Harderian gland and have correlated the porphyrin excretion resulting from B-vitamin deficiencies with that resulting from water restriction. Although the ad libitum intake of water at

Fig. 1 The pyridoxine-deficient rat on the left was maintained at 90% relative humidity for three weeks and developed red-stained fur about the head to a moderate degree. A similar pyridoxine-deficient rat on the right was maintained at 50% relative humidity and did not develop this condition.

90% relative humidity was lower than that at 50% (when vitamin-deficient diets were employed), the extent of red pigment excretion does not seem to be correlated with the absolute volume of water ingested (table 3). The gross damage to the adrenal gland as a result of pantothenic acid deficiency was not found to be related to the degree of pigment excretion. Also, the threshold of chromodacryorrhea (produced by the intraperitoneal injection of acetylcholine; Tashiro et al.,

'40) did not appear to be influenced by the humidity conditions or the vitamin deficiencies studied.

SUMMARY

1. The growth of weanling rats fed a complete diet or diets deficient in vitamin A, riboflavin, or pantothenic acid was not found to be affected by extremely low or high humidity conditions; however, animals which were deficient in pyridoxine grew more rapidly when maintained at a relative humidity of 90%.

2. The water consumption of rats receiving the complete diet and diets deficient in pyridoxine and pantothenic acid was greatest at low relative humidity.

3. At 5 to 10% relative humidity, a scaly dermatosis occurred within 12 days upon the paws of rats fed the complete diet as well as those fed diets deficient in vitamin A, riboflavin, pyridoxine or pantothenic acid. The animals fed the complete diet spontaneously recovered from their dermatosis, while those rats which remained vitamin-deficient did not recover. The addition of iodinated casein to the complete diet decreased the extent of dermatosis.

4. At 30% relative humidity, dermatosis occurred only on the paws of the vitamin-deficient animals. At and above a relative humidity of 50%, dermatosis was not observed on the paws of the vitamin-deficient rats.

5. At 90% relative humidity a red pigment was observed to occur extensively on the fur of the heads of animals fed diets deficient in pyridoxine, riboflavin and pantothenic acid. This condition did not exist at 50% relative humidity or below.

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THE INTERRELATIONSHIP BETWEEN THYROID ACTIVITY, DIETARY FAT AND LIPOGENESIS [1]

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Numerous investigations have indicated a relationship between thyroid activity and lipid synthesis or deposition. Reed, Anderson and Mendel ('32) demonstrated that induced hyperthyroidism resulted in decreased fat deposition, and Bratzler, Barnes and Swift ('49) showed that hypothyroidism resulted in animal carcasses high in fat. Other work (Anonymous, '50) has indicated the influence of thyroid substances upon cholesterol metabolism. Ershoff ('49a), Keeser ('38), Guerra ('47), Greenberg and Deuel ('50), Greenberg ('52) and many others observed the effect of fat as such, and certain fatty acids in particular, in modifying the effect of excessive thyroid activity.

It has been established that a relationship exists between dietary fat, thyroid activity and lipid deposition. It was the objective of this investigation to study the influence of hyperthyroidism, hypothyroidism, and level of dietary fat on lipid biosynthesis.

EXPERIMENTAL

Six groups of rats of the Sprague-Dawley strain were kept in individual screen-bottom cages. They were housed in an animal room maintained at 70°F. $\pm$ 2° and at 50% $\pm$ 5% hu-

[1] Taken from a thesis presented to the Graduate Faculty of Cornell University in partial fulfillment of the requirements for the Ph.D. degree.

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midity. Each group consisted of 12 weanling rats 21 days old, equally divided between the sexes.

The diets are presented in table 1. The low fat diet contained 70% sucrose and no cottonseed oil. The high fat diet contained 29.9% sucrose and 30% cottonseed oil. Two groups were fed the low fat diet and two groups the high fat diet. One group on the low fat diet and one group on the high

TABLE 1

Percentage composition of the diet

DIETARY COMPONENTS	LOW FAT	HIGH FAT
	%	%
Casein	22	30.5
Sucrose	70	29.9
Cottonseed oil	0	30.0
Salt mixture	4	5.6
Cellu flour	4	4.0
Fat supplement [1]		
Vitamin supplement [2]		
Thyroid treatment [3]		

[1] Two milliliters of corn oil (Mazola) containing 600 I.U. of vitamin A, 85 U.S.P. units of vitamin D, and 6 mg of synthetic alpha-tocopherol were administered weekly to each rat.

[2] Milligrams per 2,000 Kcal. of ration: thiamine hydrochloride, 38.0; riboflavin, 8.0; pyridoxine hydrochloride, 8.0; calcium pantothenate, 38.0; nicotinic acid, 33.0; choline chloride, 650.0; inositol, 650.0; biotin, 0.16; para-aminobenzoic acid, 27.0; folic acid, 0.1; 2-methyl-naphthoquinone, 2.7; and vitamin B_{12}, 20.0 μg.

[3] One group on the low fat diet and one group on the high fat diet received, respectively, 0.05% and 0.07% thyroid powder — U.S.P., list 25226, Control K 22507, Armour and Company, Chicago, Illinois. Two other groups of rats received low fat and high fat diets containing supplements of 0.15% and 0.21% thiouracil.

fat diet received a 0.05% and a 0.07% supplement of desiccated thyroid, respectively. A similar feeding experiment was conducted to study the effects of thiouracil. Two groups of rats received low fat and high fat diets containing supplements of 0.15% and 0.21% thiouracil, respectively.

Large supplements of the vitamins were supplied to take care of the increased demands imposed by the thyroid feeding. The diets were refrigerated at 0°C. and fed ad libitum

twice weekly. Each rat was maintained until it consumed the equivalent of 2,000 Kcal. of its particular diet. This amounted to 545 gm for the low fat diet and 391 gm for the high fat diet.[3] The differences in weight with consumption of equicaloric diets suggested a study of the metabolic rates of the rats on the various diets. The apparatus used in the determination of oxygen consumption by the rats was similar to that described by Forbes, Kriss and Miller ('34) and Kriss and Smith ('37). The oxygen consumption was calculated by subtracting the loss in weight of the animal from the CO_2 and H_2O produced. Activity was discouraged by bright illumination with two 25-watt electric light bulbs.

After the consumption of 2,000 Kcal., each rat was killed by a blow on the head; the contents of the gastrointestinal tract were removed and the entire carcass was immediately frozen at $-18°C$. It was passed through a meat chopper while still frozen, dried from the frozen state under high vacuum and ground in a Wiley mill. The Wiley mill was washed with ethyl ether after each grinding. Recovery experiments indicated less than a 0.1% loss of carcass lipid by the procedure used. Aliquots were extracted on a Bailey-Walker extractor for 24 hours with ethanol and then for 24 hours with ethyl ether. The extract was filtered and the solvent evaporated by warming in a stream of nitrogen. The residue was extracted with redistilled Skelly-solve B and made to a final volume of 100 ml. Aliquots of this extract were used for the lipid analyses.

Free cholesterol and cholesterol esters were determined by the procedure modified by Sperry and Webb ('50). Cerebrosides were determined by the method reported by Brand and Sperry ('41). The phospholipids were precipitated with acetone containing absolute ethanol saturated with $MgCl_2$. Phos-

[3] Calculated using the values: casein 4.27, sucrose 3.87, and cottonseed oil 8.84 (Kcal. per gram). Food and Agriculture Organization of the United Nations, Committee on Calorie Conversion Factors and Food Composition Tables. Energy-Yielding Components of Food and Computation of Calorie Values. Washington, D. C., 1947.

phorus was determined by the method of Sumner ('44). The determination of the phospholipid fractions depended upon the fact that the monoaminophospholipids, lecithin and cephalin, were completely hydrolyzed by 1 N KOH in 18 hours at 37°C., whereas there was no hydrolysis of sphingomyelin (Hack, '47). The total acid-soluble phosphorus in the hydrolysis mixture was used as a measure of the lecithin and cephalin present (Schmidt and associates, '46) and choline as a measure of the lecithin present (Hack, '47).

In the tables indicated, the data were subjected to analysis of variance using the F ratio (Snedecor, '46). Comparison was made between two groups and the group receiving no thyroid treatment was considered the control. When the experimental group, receiving thyroid, differed significantly, this was indicated by asterisks alongside the experimental value.

In the last part of this investigation, the study of the influence of induced hyperthyroidism on lipid deposition was extended to old rats. Discarded breeding rats of the Sprague-Dawley strain were used in this study. They were one to three years old and had been maintained at a high plane of nutrition on a stock colony ration. They were divided into 4 equal groups, on a weight basis, each group consisting of 12 rats equally divided between the sexes. Each rat was maintained, until it consumed the equivalent of 6,000 Kcal. of its diet, under conditions similar to those outlined for the young rats. The carcass was then subjected to lipid analysis.

DISCUSSION

The rats on the low fat-thyroid diet required less time to consume 2,000 Kcal. of diet than those on the low fat diet, as shown in table 2, in spite of their smaller size and maintenance requirement. The average weight gain, in grams, was significantly less in both males and females receiving thyroid on a low fat diet. Thyroid had little effect on weight gain in rats receiving the high fat diet, in confirmation of the work of Greenberg and Deuel ('50) and Greenberg ('52). This occurred even when the thyroid was added on a calorie

basis rather than on a percentage basis, as was the case in the experiment of Greenberg and Deuel ('50). The effect of thyroid on growth was quite evident with the low fat diet, in spite of the presence of large quantities of vitamin B_{12} in the diet. This is in confirmation of the work of Greenberg ('52), and lends support to the experiments of Ershoff ('49b) and Bolene, Ross and MacVicar ('50), which suggested the multiple nature of the "antithyrotoxic factor." The percentage of survival was much higher than that reported in similar experiments without the use of vitamin B_{12}. Only one rat

TABLE 2

Influence of thyroid and thiouracil on rate and efficiency of weight gain

	LOW FAT	LOW FAT-THYROID	LOW FAT-THIOURACIL	HIGH FAT	HIGH FAT-THYROID	HIGH FAT-THIOURACIL
	Average days required to consume 2,000 Kcal.					
Males	42.6	43.3	47.3	41.8	39.8	49.5 [1]
Females	44.4	44.2	50.9	41.8	41.0	58.7 [1]
	Average gain in grams on consumption of 2,000 Kcal.					
Males	155.1	123.6 [2]	157.4	161.1	151.5	126.6 [1]
Females	141.5	112.2 [1]	103.5 [1]	143.5	147.1	83.5 [2]

[1] Significant difference at 5% level.
[2] Significant difference at 1% level.

(male, receiving the low fat-thyroid diet) of the 72 used in this experiment did not survive.

Feeding thiouracil caused a reduction in the daily caloric intake, as is indicated in table 2. The rats fed thiouracil required almost 20% longer to consume 2,000 Kcal. The average gain in grams on the consumption of 2,000 Kcal. was lower in the thiouracil-fed rats, except in the case of the males on the low fat diet. This may be accounted for by the maintenance requirement over a longer period of time. Thiouracil was shown to exert a greater effect on the high fat diet than on the low fat diet.

The effect of thyroid feeding on the heat production of rats is demonstrated in table 3. Each figure is the average for 4 representative rats (two males and two females) fasted 24 hours. The rectal temperatures of the rats receiving thyroid were all above average, as is shown in table 3, although the increase was most pronounced in the rats receiving the low fat diet. The rise in body temperature was an indication of an increased metabolism and closely paralleled the oxygen consumption. The rectal temperatures and oxygen consumption indicated a reduced heat production in animals on the high

TABLE 3

The effect of 40 days' thyroid or thiouracil feeding on the heat production of young rats

	LOW FAT	LOW FAT-THYROID	LOW FAT-THIOURACIL	HIGH FAT	HIGH FAT-THYROID	HIGH FAT-THIOURACIL
Male						
Rectal temperature °F.	101.6	103.7	100.3	101.0	101.8	98.9
ml O_2 used/hour /100 gm body weight$^{3/4}$	103	151	81	93	116	78
Female						
Rectal temperature °F.	101.4	103.6	99.8	101.2	101.9	98.5
ml O_2 used/hour /100 gm body weight$^{3/4}$	97	152	84	85	109	75

fat diet. It appeared that a high fat diet tended to suppress the increased metabolic rate of hyperthyroidism. Heat production, as reported in table 3, was decreased by thiouracil feeding as evidenced by rectal temperature and oxygen consumption.

In view of the known diabetogenic effect of thyroid with relation to carbohydrate metabolism (Houssay, '48), a preliminary observation was made with respect to the possible presence of glycosuric urine in the various groups. Urine collections were made for a 24-hour period from two male rats, after 40 days of feeding, from the low fat, low fat-

thyroid and high fat-thyroid groups. Only the low fat-thyroid group showed the presence of sugar in the urine, according to the method of Sumner ('25), and this was of a mild nature. This suggests an increase in the effect of a high carbohydrate diet on thyroid-fed rats in producing diabetes.

Carcass analyses of the young animals, presented in table 4, showed that the principal difference among the animals fed thyroid was in the neutral fat fraction. The term "essential lipid" was used to denote the lipid constituents comprising the cerebrosides, cholesterols and phospholipids. This fraction was higher in the rats on the high fat diet. Cholesterol, and especially the cholesterol esters, tended to be lower in the thyroid-fed animals, although there was little difference between the rats fed a low fat diet and those fed a high fat diet. The percentages for the males and females were averaged in the preparation of table 4, since no sex difference was observed.

TABLE 4

Influence of thyroid and thiouracil on the percentage lipid composition of the young rat carcass (dry weight basis)

	LOW FAT	LOW FAT-THYROID	LOW FAT-THIO-URACIL	HIGH FAT	HIGH FAT-THYROID	HIGH FAT-THIO-URACIL
Total lipid	28.38	20.66[1]	35.67[1]	31.72	27.02	50.52[1]
Neutral fat	23.32	15.07[1]	30.40[1]	25.91	21.18	45.31[1]
Essential lipid	5.06	5.59	5.27	5.81	5.84	5.21
Cerebrosides	0.97	1.21	1.02	1.16	1.25	1.01
Free cholesterol	0.30	0.28	0.27	0.33	0.31	0.23
Cholesterol esters	0.34	0.26[2]	0.29	0.31	0.30	0.30[2]
Phospholipid	3.46	3.84	3.69	4.01	3.98	3.67[2]
Cephalin	1.60	1.80	1.74	2.01	1.98	1.75
Lecithin	1.43	1.60	1.52	1.55	1.60	1.51
Sphingomyelin	0.43	0.44	0.43	0.45	0.40	0.41
Water	68.53	68.97	64.70	66.37	67.81	57.47

[1] Significant difference at 1% level.

[2] Significant difference at 5% level.

The lipid composition data with respect to the rat carcass presented in table 4 indicate that the total lipid content of the rat carcass is increased by thiouracil feeding. The increase is accounted for largely in the neutral fat fraction. Thiouracil tended to cause a significant increase in the per cent of cholesterol esters, which was apparent when the percentage composition was placed on a dry, neutral fat-free basis. The apparent decrease in phospholipid may be partly attributed to the tendency of the larger amount of neutral

TABLE 5

Influence of thyroid on the percentage lipid composition of the old rat carcass (dry weight basis)

	LOW FAT	LOW FAT-THYROID	HIGH FAT	HIGH FAT-THYROID
Total lipid	37.81	33.13 [1]	43.26	40.79
Neutral fat	33.10	28.16 [1]	38.58	35.80
Essential lipid	4.71	4.97	4.68	4.99
Cerebrosides	1.50	1.59	1.62	1.69
Free cholesterol	0.30	0.35	0.29	0.33
Cholesterol esters	0.24	0.28	0.21	0.23
Phospholipid	2.67	2.75	2.56	2.74
Cephalin	1.45	1.54	1.43	1.60
Lecithin	0.94	0.92	0.93	0.88
Sphingomyelin	0.28	0.29	0.20	0.26
Water	58.62	60.76	58.41	59.67

[1] Significant difference at 5% level.

fat to dilute out the other lipid fractions. This may also be demonstrated by calculating the percentages on a neutral fat-free basis. Under these conditions the phospholipid appeared to increase in the rats on the high fat-thiouracil diet, although the difference was not significant. The results are in general agreement with those reported by Bratzler, Barnes and Swift ('49), who used a paired-feeding technique.

The old rats were not as sensitive to thyroid feeding as the young rats. The old rats on the high fat diet showed a much greater weight gain than those on the low fat diet. This may be accounted for on the basis of greater palatability

of the high fat diet, although a similar effect was not observed in younger rats. The essential lipid pattern was not altered significantly by thyroid feeding or level of fat in the diet, as is indicated in table 5. This indicates a greater stability of the essential lipid fraction in the rat after maturity.

A comparison of the carcass composition of the old rats (table 5) with that of the young rats (table 4) demonstrates that aging is associated with an increase in neutral fat and a decrease in essential lipid. The phospholipid fraction decreases, while the cerebroside fraction increases with age. The changes in the percentages of the lipids are a reflection of the varying percentages of muscular, nerve, and adipose tissue.

SUMMARY

Immature rats receiving a low fat diet and thyroid showed retarded growth, elevated body temperature, increased oxygen consumption and mild glycosuria. Lipid analysis of their carcasses revealed a reduced total lipid content which was accounted for largely in the neutral fat fraction. The cholesterol ester was the only fraction of the essential lipid which was decreased significantly. All of these effects of hyperthyroidism in the young rats were alleviated by a diet containing 30% cottonseed oil. The old rats were not as sensitive to thyroid feeding as the young rats and only the neutral fat fraction was modified.

Young rats receiving thiouracil showed retarded growth, decreased body temperature and decreased oxygen consumption. The influence of thiouracil was more apparent on the high fat diet than on the low fat diet. Thiouracil feeding resulted in large increases in the percentage of total lipid deposited. This was accounted for largely in the neutral fat fraction. A small increase in the per cent of cholesterol esters was the only change observed in the "essential lipid" fraction under the experimental conditions used in this investigation.

In general, the effects of induced hyperthyroidism were reversed in hypothyroidism. A high fat diet tended to alleviate the effects of increased thyroid activity but enhanced the characteristics of depressed thyroid activity.

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THE EFFECT OF HEAT TREATMENT ON THE NUTRITIVE VALUE AND HEMAGGLUTINATING ACTIVITY OF SOYBEAN OIL MEAL[1]

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ONE FIGURE

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The beneficial effect of proper heat treatment on the nutritive value of the soybean protein is well known (see review by Liener, '50). Any simple test which can be substituted for long and costly animal experiments as a means of differentiating meals which are nutritionally inadequate because of either underheating or overheating is a matter of considerable practical interest. Several such tests which have been proposed in the past include the determination of urease activity (Caskey and Knapp, '44), soluble nitrogen (Bird et al., '47), antitryptic activity (Westfall and Hauge, '48; Borchers et al., '48), enzymic release of amino acids (Riesen et al., '47), and fluorescence (Balloun and Johnson, '52). Recent studies, however, would seem to indicate that none of these tests alone is completely satisfactory for detecting overheated as well as underheated soybean oil meals (Simon and Melnick, '50; Clandinin and Robblee, '52; Balloun et al., '51).

Previous work undertaken in this laboratory in an attempt to elucidate the mechanism whereby heat treatment enhances the nutritive value of soybean oil meal has led to the isolation

[1] Paper 2946, Scientific Journal Series, Minnesota Agricultural Experiment Station.

from unheated soybean flour of a heat-labile protein fraction which is toxic to rats when injected and retards growth when fed orally (Liener, '53; Liener and Pallansch, '52). A characteristic property of this toxin, which was given the name "soyin" [2], is its marked *in vitro* agglutinating action toward the red blood cells of the rabbit. If the destruction of soyin by heat were found to parallel the improvement in the nutritive value of the soybean protein, a simple test would be available for detecting inadequately heated soybean oil meals. This paper presents evidence supporting the feasibility of such a test. Furthermore, it was found that by the simple expedient of treating the red blood cells with papain, the sensitivity of the test could be increased to the point where overheated samples of soybean oil meal could likewise be detected.

EXPERIMENTAL

Preparation of soybean oil meal samples

All heated samples were prepared from the same lot of defatted soybean oil meal,[3] which, according to the manufacturer, had been subjected to a minimum amount of heat treatment. The meal was spread in enamel pans in a layer not exceeding one inch in thickness and autoclaved either at atmospheric pressure (autoclave door ajar) or at 15 pounds' pressure for various periods of time. After being dried overnight in a blast of warm air, the autoclaved samples were ground to a fine powder in a hammer mill.

Growth studies with chicks

Day-old, male, New Hampshire chicks were distributed into groups of equal weight and placed in electrically-heated batteries with raised screen floors. The chicks were fed ad libitum and fresh water was given daily. The basal diet con-

[2] Pending proof of their non-identity, the terms "soyin" and "hemagglutinin" will hereafter be used interchangeably.

[3] Nutrisoy XXX, Archer-Daniels-Midland Company, Minneapolis, Minnesota.

sisted of soybean oil meal at a level supplying 20% protein, 4.0% clarified corn oil, 6.0% salts 3 M,[4] 0.6% DL-methionine, 0.2% choline chloride, and the following amounts of vitamins per kilogram of ration: calcium pantothenate, 20 mg; thiamine hydrochloride, 20 mg; riboflavin, 8 mg; pyridoxine hydrochloride, 6 mg; biotin, 0.2 mg; nicotinic acid, 100 mg; folic acid, 2 mg; alpha-tocopherol acetate, 10 mg; menadione, 1 mg; vitamin A, 20,000 I.U.; vitamin D_3, 750 I.U.; and vitamin B_{12}, 10 μg. All diets were adjusted to 100% with glucose (cerelose).

Hemagglutinating activity

To 5 gm of the soybean sample [5] in a stoppered bottle were added 50 ml 0.9% NaCl, and the suspension was shaken vigorously for one minute. Although extraction of the hemagglutinin is complete after allowing the suspension to stand for one hour, it was generally convenient to set the samples in the refrigerator overnight. A clear yellow solution was usually obtained either by centrifuging or filtering the suspension. Some soybean samples yielded a cloudy supernate or filtrate, but this did not seriously interfere with the subsequent hemagglutinating test.

A suspension of red blood cells from rabbit's whole blood [6] was freshly prepared in the following manner. The hematocrit value (H) was determined on a portion of the whole blood, using a hematocrit tube (Sanford and Magath, '29). One volume of the whole blood (V_b) was diluted with 4 volumes of cold 0.9% saline, centrifuged at 2,000 R.P.M. for 10 minutes, and the supernatant fluid discarded. The sedimented cells were washed with saline in this manner at least three times, or until the supernatant fluid was colorless. The sedimented

[4] Salts 2 M of Hill and Briggs ('51) modified by replacing the ferric ammonium citrate with ferrous ammonium sulfate at an equivalent level of iron.

[5] Preliminary experiments had shown that it was not necessary to grind the samples into a fine powder before running the hemagglutination test.

[6] Collected by heart puncture in an oxalated tube. Citrated rabbit blood, which may be obtained by air express from Carworth Farms, Inc., New City, New York, was also found to be quite satisfactory for this purpose.

cells were finally suspended in that volume of 0.9% saline (V_{s1}) Which, based on the hematocrit value of the original whole blood, would give a final red blood cell concentration of 4%.[7]

Berger ('52) has reported that it is possible to increase the sensitivity of red blood cells to agglutination with ricin by first treating the cells with papain. This observation led to the adoption of the following procedure which, as will be noted later, markedly increased the sensitivity of the hemagglutinating test described here. Sufficient 0.9% saline (V_{s2}) was added to a known volume of the whole blood (V_b) to give a final red blood cell concentration of 5%.[8] To 4 volumes of the diluted blood was added one volume of a 2% solution of papain[9] in M/20 phosphate buffer of pH 5.9, and the mixture was incubated at 37° for one hour. The cells were separated by centrifugation, washed repeatedly with saline as described in the previous paragraph, and finally suspended in sufficient saline to give a final cell concentration of 4%.[10]

In performing the hemagglutinating test, decreasing volumes of the original soybean extract, or a suitable dilution thereof with 0.9% saline, were added to a series of tubes (10 × 75 mm), usually 10 in number. To each tube containing 0.5 ml of diluted extract was added 0.2 ml of the 4% suspension of either the untreated or the papain-treated cells. The tubes were incubated at 37° for one hour, at room temperature for two to 4 hours, or in the refrigerator overnight. Agglutination was read by tapping the tube and noting the clumping of the cells. Cells which had not been agglutinated dispersed readily to form a uniform suspension. A tube containing 0.5 ml saline plus 0.2 ml of the cell suspension served

[7] V_{s1} is readily calculated from the formula $\frac{V_b H}{4}$. This suspension of red blood cells should be kept in the refrigerator to minimize hemolysis and, before reusing, should be washed with successive portions of cold saline until the supernate is colorless. Excessively hemolyzed blood should be discarded.

[8] $V_{s2} = \frac{V_b (H-5)}{5}$.

[9] Parke-Davis.

[10] See footnote 7.

as a negative control for this purpose. Positive agglutination was indicated by the formation of clumps of cells which did not disperse but immediately settled to the bottom of the tube. The degree of agglutination was measured by the size of the cell aggregate and could be arbitrarily graded on this basis from zero to 4 +. In figure 1 is shown a photograph of tubes displaying the typical agglutinating action which one observes.

If one hemagglutinating unit (HU) is defined as the least amount of hemagglutinin which will produce positive evidence of agglutination (1 +) under the conditions specified herein, it is possible to calculate the hemagglutinating activity of the original soybean meal as follows:

$$\text{HU/gm} = \frac{D_a \times D_b \times S}{V} = 20 \times D_a \times D_b,$$

where

V = volume of extract in tube 1 (= 0.5 ml),
D_a = dilution factor of extract in tube 1 (= 1 unless original extract was diluted),
D_b = dilution factor of tube containing 1 HU, and
S = ml original extract/gm soybean meal (= 10).

The detailed data relating to several typical determinations which illustrate the method for computing hemagglutinating activity are presented in table 1.

RESULTS

The influence of heat treatment of soybean meal on hemagglutinating activity and nutritional value as measured by chick growth is summarized in table 2. With both the untreated and papain-treated cells, the degree to which the hemagglutinin was destroyed by heat was accompanied over a rather wide range by a corresponding increase in the growth-promoting value of the soybean protein. The use of papain-treated cells markedly increased the sensitivity of the hemagglutinating test. Several samples which were negative when tested with the untreated cells gave a distinctly positive reaction with the papain-digested cells.

A closer examination of the data in table 2 permits the following conclusions: (a) hemagglutinating activity, using

either the untreated or papain-treated cells, provides a valid index of the degree to which soybean oil meals may have been inadequately heated; (b) the use of the untreated cells does not serve to differentiate the overheated samples from those which have received optimum heat treatment; and (c) only those samples which have received excessive heat treatment give a negative hemagglutinating reaction with the papain-treated cells. While these results indicate that the

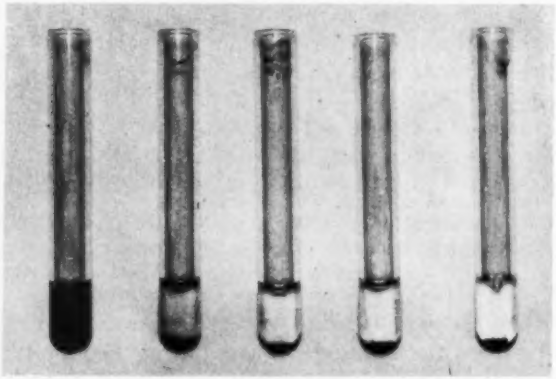

Fig. 1 Photograph of tubes displaying various degrees of hemagglutination under the conditions specified in the text. From *right* to *left*, the tubes are arranged in order of increasing dilutions of a saline extract of soybean oil meal, which in this instance is the unheated sample. The dilutions with the corresponding agglutination scores are as follows: tube 1 — none, 4 +; tube 2 — 1: 8, 3 +; tube 3 — 1: 32, 2 +; tube 4 — 1: 64, 1 +; tube 5 — 1: 128, 0. The end-point tube in this series, therefore, is tube 4.

use of papain-treated cells would be preferred because of its ability to detect overheated samples as well as those which have been inadequately heated, a differentiation between underheated and properly heated samples would depend solely on quantitative differences in hemagglutinating activity. A serious objection to such a test then would be the reliance that would have to be placed on an absolute value for hemagglutinating activity. A much more desirable test would be one in which a qualitative difference, that is the presence or

absence of hemagglutinating activity, could be used to distinguish between samples receiving proper or improper heat treatment.

A comparison of the results obtained with both types of cells reveals a rather interesting qualitative relationship.

TABLE 1

Typical determinations of hemagglutinating activity of soybean oil meal samples, using untreated and papain-digested red blood cells from the rabbit

SOYBEAN MEAL SAMPLE		UNHEATED		AUTOCLAVED AT ATM. PRESSURE FOR 45 MIN.		AUTOCLAVED AT 15 LB. PRESSURE FOR 15 MIN.	
Treatment of red blood cells		None	Papain	None	Papain	None	Papain
Tube	D_a [1]	1	1:25	1	1:10	1	1:5
	D [2]	Hemagglutinating score					
1	1	4+	4+	4+	3+	1+ [3]	4+
2	1: 2	3+	3+	3+	3+	0	3+
3	1: 4	3+	3+	2+	2+	0	2+
4	1: 8	3+	3+	1+ [3]	1+ [3]	0	1+ [3]
5	1: 16	2+	2+	0	0	0	0
6	1: 32	2+	1+ [3]	0	0	0	0
7	1: 64	1+ [3]	0	0	0	0	0
8	1:128	0	0	0	0	0	0
9	1:256	0	0	0	0	0	0
10	1:528	0	0	0	0	0	0
HU/gm [4]		1,280	16,000	160	1,600	20	800

[1] D_a = dilution of original extract in tube 1.

[2] D = subsequent dilution of extract in tube 1.

[3] Dilution equivalent to one hemagglutinating unit = D_b. Some variation in reading the end-point may be expected in replicate determinations but the difference was never greater than one tube (factor of two).

[4] Given by the formula $20 \times D_a \times D_b$. See text for derivation.

Those samples which gave maximum growth response (atmospheric pressure for 75, 90, 150 minutes; 15 pounds' pressure for 20 minutes) were also those that gave no hemagglutinating response with the untreated cells but were distinctly reactive toward the papain-treated cells. (The only exception was the sample which had been heated at 15 pounds'

pressure for 15 minutes. This sample had a small but positive hemagglutinating action toward the untreated cells as well.) All those samples which were found to be nutritionally inferior because of underheating (atmospheric pressure

TABLE 2

Summary of hemagglutinating and chick growth data of soybean oil meal samples subjected to various degrees of heat treatment

HEAT TREATMENT OF SOYBEAN MEAL	CHICK WEIGHT IN 16 DAYS [1]	HEMAGGLUTINATING ACTIVITY [2]	
		Untreated cells	Papain-treated cells
min.	*gm*	*HU/gm*	*HU/gm*
Atmospheric pressure			
0 (inadequate)	90 ± 10.2	1,280	16,000
10 (inadequate)	89 ± 11.6	1,280	16,000
30 (inadequate)	138 ± 12.3	320	2,400
45 (inadequate)	151 ± 13.3	160	1,600
60 (inadequate)	157 ± 7.1	80	800
75 (optimum)	172 ± 8.0	0	400
90 (optimum)	173 ± 9.6	0	400
150 (optimum)	168 ± 6.8	0	400
15 lb. pressure			
5 (inadequate)	122 ± 12.3	480	3,200
15 (optimum)	159 ± 7.6	20	800
20 (optimum)	168 ± 4.7	0	400
30 (excess)	152 ± 8.6	0	0
120 (excess)	67 ± 5.0	0	0

[1] Mean ± standard error of the mean based on 8 chicks per group. Analysis of variance (Snedecor, '46) revealed that the least significant difference between treatments at a probability level of 5% was ± 15.0 gm. The growth response given by the sample heated at atmospheric pressure for 90 minutes was considered the maximum, and, on this basis, samples were classified into one of three groups, as indicated in parentheses in the first column.

[2] Based on the results of at least three replicate runs. Variations in the end-point tube which were occasionally encountered (see footnote 3, table 1) were not included in the calculation of hemagglutinating activity.

for zero, 10, 30, 45, and 60 minutes; 15 pounds' pressure for 5 minutes) gave a positive reaction with both the treated and untreated cells, and the two samples which were heated to excess (15 pounds' pressure for 30 and 120 minutes) gave a negative reaction with both types of cells.

A rather simple qualitative technique, therefore, appeared to be at hand for differentiating underheated, optimally heated, and overheated samples of soybean oil meal. The reliability of this test was assessed by conducting growth studies and both hemagglutinating tests on several commercial samples of soybean oil meal selected at random on the University Farm. Examination of the data in table 3 shows that the results of this test are in accord with previous ob-

TABLE 3

Growth-promoting value and hemagglutinating activity of commercial samples of soybean oil meals

SAMPLE	NO. OF CHICKS PER GROUP	WEIGHT AT 16 DAYS [1]	HEMAGGLUTINATING ACTIVITY [2]	
			Untreated cells	Papain-treated cells
		gm.		
A	8	178 ± 9.1	—	+ (1,600)
B	6	161 ± 7.8	—	—
C	9	153 ± 3.8	—	—
D	9	165 ± 5.8	—	+ (400)
E	9	148 ± 9.3	—	—
F	10	127 ± 8.8	+ (640)	+ (12,800)
G	9	151 ± 6.3	—	—

[1] Mean ± standard error of the mean. Analysis of variance (Snedecor, '46) revealed that the least significant difference between samples at a probability level of 5% was ± 16.0 gm.

[2] —, no hemagglutinating action could be detected.
+, hemagglutinating action was observed. Activity in terms of hemagglutinating units/gram is indicated in parentheses.

servations. Samples A and D were nutritionally superior to the other samples tested, a fact which could have been predicted from the hemagglutinating data, namely a negative test with the untreated cells and a positive reaction with the papain-treated cells. Likewise the nutritional inferiority of sample F, presumably due to inadequate heat treatment, would have been expected on the basis of the positive reaction obtained with both types of cells. The remaining samples, B, C, E and G, also failed to produce maximum growth response, a fact which is consistent with the negative reaction that is

obtained in both tests with excessively heated samples of soybean meal.

DISCUSSION

Although hemagglutinating activity may provide a useful index of proper heat treatment, it is not to be inferred that the hemagglutinating factor is necessarily the principal cause of the poor growth obtained with chicks fed raw soybean protein. If hemagglutinating activity can be assumed to be a reflection of the proportion of the crude soybean protein which remains in the raw form after heat treatment, it follows that the plotting of growth as a function of hemagglutinating activity is in effect equivalent to plotting growth as a function of the raw soybean content of the diet. If the data of table 2 are plotted in this manner, the curves obtained are similar to the ones reported by Almquist and Merritt ('52). These authors noted that the growth inhibition of chicks fed increasing proportions of raw soybean developed abruptly at first and thereafter decreased as the quantity of raw soybean was increased. Our results could be interpreted, therefore, to support the view advanced by these authors that soybean antitrypsin inactivates the trypsin secreted by the chick but permits the remaining proteolytic system to function at a lower degree of efficiency. Still to be explained, however, is the further slight inhibition of growth that occurs with increased proportions of raw soybean subsequent to this large initial depression in growth response. Whether or not soyin, the substance believed to be identical with the hemagglutinin, is responsible for any of the inhibitory effect exerted by raw soybeans on chick growth must await studies involving the feeding of purified soyin to chicks, such as those recently reported for rats (Liener, '52).

SUMMARY

A procedure for determining the hemagglutinating activity of soybean oil meals is described in detail. This method involves determination of the highest dilution of a saline ex-

tract of the soybean oil meal which will agglutinate the red blood cells of the rabbit under specified conditions. Previous digestion of the red blood cells with papain markedly enhances the sensitivity of this test. The decrease in hemagglutinating activity paralleled over a wide range the increase in the nutritive value of soybean protein effected by proper heat treatment. Although the use of either the untreated or papain-treated cells permitted a quantitative estimate of the degree of underheating of soybean oil meal, only the latter method was satisfactory for detecting samples which had been overheated. When the results of one test were considered in conjunction with those of the other, however, it was observed that optimally heated samples gave a negative reaction with the untreated cells and a positive reaction with the papain-treated cells. Underheated samples gave a positive and overheated samples a negative reaction with both types of cells. This rapid qualitative technique proved reliable in assessing the adequacy of heat treatment of 6 samples of commercial soybean oil meal which were tested.

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EFFECT OF ANTIBIOTICS ON RATS FED AMINO ACID-SUPPLEMENTED RICE DIETS

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INTRODUCTION

Pecora and Hundley ('51) reported that a 90% white polished rice diet was markedly improved by the addition of lysine and threonine, as judged by growth response in young rats. It was also noted that this same diet supplemented with other essential amino acids, individually or in all the possible combinations of pairs, or with many of the possible combinations of three and 4, was not further improved. In view of the many recent reports indicating that certain antibiotics promote growth, experiments were made to determine whether antibiotics exert any effect on rice diets, with or without amino acid supplements, particularly since these diets supply only 5.4% protein of vegetable origin.

Penicillin, aureomycin, streptomycin, terramycin, bacitracin and chloromycetin were tested in these experiments. All except chloromycetin significantly improved a 90% rice diet, whereas only penicillin and aureomycin improved the lysine-threonine-supplemented rice diet.

EXPERIMENTAL METHODS

White polished rice of commercial grade bought in the open market and ground to 60–80 mesh was used in all these experiments. Test diets were prepared so that the sum of the

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different essential amino acid supplements, antibiotics and rice totaled 90%. Fat, salt mixture and vitamins made up the rest. The basal rice diet (R) was composed of 90% rice, 4% salts, 5% fat and 1% vitamin powder. The diet composition and the amount of amino acids added, except for lysine and threonine, were the same as those reported earlier (Pecora and Hundley, '51). The diets contained adequate amounts of all known nutrients except for protein, which was supplied solely by the rice at approximately a 5.4% level. The several lots of rice used in these experiments contained 1.01 to 1.06% nitrogen. The stock [2] diet was analyzed as containing 4.18% nitrogen. Each essential amino acid was added to the experimental rice diets in the amount suggested by Rose ('37) for the growing rat (except where otherwise stated), making allowance for the inactive enantiomorphs. Antibiotics [3] were added to the diets at a level of 20 mg %.

Male litter-mate Sprague-Dawley rats from the National Institutes of Health colony, about 24 days old, average weight about 46 gm, were distributed equally into experimental groups. They were housed individually in elevated wide mesh wire-bottom cages, and were fed the experimental diets and water ad libitum. All tests were continued for at least 9 weeks. Animals receiving a stock diet [4] were included in each phase of the experiment as an aid in establishing relative rates of growth. A preliminary report of some of these findings was made earlier (Pecora, '52).

RESULTS AND DISCUSSION

Lysine-threonine-supplemented rice diet

Penicillin and aureomycin produced definite growth stimulation when added to the basal diet containing 2.00% DL-lysine and 1.20% DL-threonine (diet 2624), while the remainder of

[2] Purina Dog Chow.

[3] Crystalline penicillin G sodium, crystalline aureomycin HCl, crystalline terramycin HCl, streptomycin (calcium chloride complex), bacitracin powder and crystalline chloromycetin.

[4] See footnote 2.

the antibiotics were without effect.[5] It was observed earlier that a supplement of one-fifth the amount of lysine and threonine (used in diet 2624) would give a growth response equal to that obtained with a full supplement. Somewhat unexpectedly, it was found that the antibiotics penicillin and aureomycin were more effective when the smaller quantities of DL-lysine, 0.40%, and DL-threonine, 0.24%, were used (diet Rlt). This diet supplemented with penicillin produced 20 to

TABLE 1

Effect of antibiotic, vitamin B_{12} and urea on growth of young rats fed a lysine-threonine-supplemented rice diet

DIET	NO. OF RATS	AVERAGE GROWTH INCREMENTS PER RAT AT		
		3 wks.	6 wks.	9 wks.
		gm	*gm*	*gm*
Stock	20	93.9	175.2	222.0 ± 6.4 [1]
Rlt [2]	20	36.1	75.0	124.8 ± 4.3
Rlt + vitamin B_{12} [3]	10	35.0	72.0	125.4 ± 5.1
Rlt + aureomycin	20	44.4	87.1	140.4 ± 3.4
Rlt + aur. + B_{12}	10	51.0	103.5	159.9 ± 7.8
Rlt + penicillin	20	46.2	98.2	156.5 ± 5.5
Rlt + pen. + B_{12}	10	47.6	98.3	157.1 ± 9.0
Rlt + urea	5	44.0	86.8	126.2 ± 11.0
Rlt + urea + vit. B_{12}	5	48.2	106.4	163.6 ± 10.1

[1] Standard error of the mean = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[2] Basal rice diet plus DL-lysine HCl 0.40% and DL-threonine 0.24%.

[3] Twenty micrograms of vitamin B_{12}/kilogram.

35%, and with aureomycin 15 to 25%, increases in growth over the control diet in 9 weeks. These increases were obtained consistently, and are highly significant statistically ($p = < 0.01$). Table 1 presents a representative experiment showing the typical responses which were consistently obtained with aureomycin and penicillin over the past year. Pooled values for growth increments at 9 weeks from 12 different experiments representing about 80 rats on each diet gave the following means: rice, lysine and threonine 119.3 gm,

[5] An earlier report (Pecora, '52) unintentionally omitted the fact that penicillin also gave growth stimulation with this diet.

with aureomycin 146.5 gm, and with penicillin 153.7 gm. L-lysine (0.20%) and L-threonine (0.12%) tested in some of the experimental diets gave the same response as that obtained with the DL forms in the amount used in diet Rlt. D-threonine was found to be inactive either with or without antibiotics. D-lysine was not available for testing.

Neither diet Rlt nor the same diet plus penicillin was improved by the addition of vitamin B_{12} (20 μg per kilogram), as judged by growth response (table 1). However, the addition of vitamin B_{12} to diet Rlt plus aureomycin increased the growth response from 140.4 gm to 159.9 gm per rat in 9 weeks, thus equalling the growth obtained with penicillin. Vitamin B_{12} did not improve growth in the basal diet (R) nor did it alter growth using the basal diet plus several amino acid combinations.[6] However, 2% urea and vitamin B_{12} added to diet Rlt produced an increase in growth of 39 gm over that obtained with diet Rlt (124 to 163 gm, $p = < 0.01$). No growth stimulation was obtained with urea when vitamin B_{12} was omitted. Antibiotics were not tested with diet Rlt and urea. The fact that antibiotics gave better growth with the smaller quantities of lysine and threonine suggests that the amino acid balance of the rice protein may be very sensitive to small changes in amino acid concentration. It further suggests that the increased growth obtained with this type of diet is not dependent solely on extra nitrogen, since the addition of amino acids other than lysine and threonine with the same or more nitrogen was ineffective even when vitamin B_{12} was added. It is of interest to note that the rice-lysine-threonine diet plus aureomycin or penicillin, and also diet Rlt plus urea and vitamin B_{12}, produced growth approximately equal to that obtained with a similar strain of rats fed a 15% casein diet by other investigators in our laboratories at the National Institutes of Health (Schwarz, '52; Hundley, '52).

[6] Lysine, histidine, threonine, lysine and threonine or lysine and histidine were used as supplements in these experiments.

Basal rice diet

The basal rice diet (R) was significantly improved, as judged by growth response, by the addition of each of the antibiotics tested except chloromycetin. The average weight gain of 27 gm for 9 weeks for the controls was increased by at least 35% in all instances. These results were obtained on two different occasions, using 5 rats per group. An effort was made further to improve the basal rice diet plus aureomycin by the addition of different combinations of amino acids that might be limiting growth. Lysine alone and lysine plus each of the remaining essential amino acids individually produced no better growth than the basal diet plus aureomycin, except for the combination of lysine and threonine. Similarly, histidine or lysine and histidine supplemented with each of the remaining essential amino acids individually produced no better growth than the basal diet plus aureomycin, except for the combination lysine, histidine and threonine, which produced the same response as lysine and threonine. Antibiotics other than aureomycin were not tested in this series of experiments.

Organ: body weight ratios

It was of interest to determine if the addition of antibiotics to diet Rlt produced any alterations in the weight of some of the vital organs. Organ: body weight ratios for several organs were determined in one series of animals after 9 weeks on the experimental diets. The average values for the liver, kidneys, total gastrointestinal tract, cecum, spleen, heart, adrenals, testes and thymus of the rats fed diet Rlt plus antibiotics were similar to those of animals receiving the control diet Rlt. On the other hand, these values were quite different from those obtained from the stock-fed group. Rats fed the control diet Rlt had considerably smaller organ: body weight ratios for the gastrointestinal tract, cecum, liver and kidneys, and considerably larger ratios for the heart, testes and thymus, than the stock-fed group. The values for the

spleen and adrenal were the same for all groups (table 2). The average nose to anus length of rats fed the control diet Rlt was 189 mm, compared to 194 mm for the animals receiving antibiotics. The average carcass [7] weights of the antibiotic groups were approximately 30 gm greater than those of the controls on diet Rlt (table 3). These facts suggest that the increased gain in body weight in the antibiotic-fed rats was of essentially normal tissue composition. However, carcass analyses for fat, protein, water and ash would be required to permit any firm conclusions as to the nature of the increased tissue due to antibiotics. Microscopic observations of some of our animals receiving antibiotics have shown a slight increase in tissue fat and glycogen but showed no abnormalities otherwise. It would be of prime interest at this time, especially with the wide use of antibiotics in animal feed as a means of stimulating growth and of increasing body size, to know whether such animals have carcasses similar in composition to those of animals raised in the conventional manner.

Antibiotics and growth pattern

One series of rats kept on diet Rlt with antibiotics for 30 weeks continued to maintain the extra weight which was gained during the first 10 weeks on the diet. The characteristic growth pattern was as follows: The greatest rate of growth occurred during the first 10 weeks after weaning, with the antibiotic group growing at a faster rate of approximately 2.5 gm/rat/day vs. 2.0 gm/rat/day for the controls on diet Rlt. Growth rate diminished considerably during the next three weeks, with the antibiotic group still growing at a faster rate (approximately 1.8 gm/rat/day vs. 1.5 gm/rat/day). From then on, the growth rates were about the same.

[7] The term "carcass" in this report denotes an animal with the following organs removed: thymus, lungs, heart, liver, total gastrointestinal tract, spleen, adrenals, kidneys and testes.

TABLE 2

Average organ : body weight ratios of rats fed the lysine-threonine-supplemented rice diet plus antibiotics for 9 weeks[1]

DIET	NO. OF RATS	ORGAN : BODY WEIGHT (%)								
		Total G.I. tract	Cecum	Testes	Thymus	Liver	Kidney	Spleen	Heart	Adrenals
Stock	10	9.74	2.45	1.09	0.164	4.24	0.77	0.35	0.31	0.019
Rlt	9	6.54	0.85	1.37	0.193	4.03	0.62	0.34	0.36	0.019
Rlt + aureomycin	10	6.02	0.74	1.30	0.197	3.69	0.62	0.29	0.35	0.019
Rlt + penicillin	10	6.51	0.84	1.23	0.219	3.95	0.62	0.31	0.31	0.020
Rlt + terramycin	10	6.20	0.78	1.31	0.199	3.97	0.74	0.29	0.35	0.021

[1] Average weight of rats, 46.2 gm; average age, 23 days at start of experiment.

TABLE 3

Growth increments, carcass weights and nitrogen balance determination of rats fed a lysine-threonine-supplemented rice diet plus antibiotics

DIET	NO. OF RATS [1]	AVE. GROWTH INCREMENTS (9 wks.)	AVE. CARCASS WT. (9 wks.)	a AVE. N INTAKE/ RAT	b AVE. FECAL N/RAT	c AVE. URINE N/RAT	N BALANCE = a−(b+c) [2]	PROTEIN EFFICIENCY [3]
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	
Stock	10	241.9 ± 6.4 [4]	228.4	2.698	0.622	1.511	0.565	0.91
Rlt	9	129.1 ± 4.4	146.4	0.472	0.067	0.151	0.254	2.13
Rlt + aur.	10	161.5 ± 4.6	175.8	0.463	0.052	0.168	0.243	2.84
Rlt + pen.	10	159.0 ± 5.5	174.0	0.482	0.056	0.184	0.242	2.50
Rlt + terr.	10	136.4 ± 6.1	153.0	0.426	0.044	0.136	0.246	2.32

[1] Average weight of rats, 46.2 gm; average age, 23 days at start of experiment.
[2] Nitrogen balance studies made for a 4-day period during the 5th week on experimental diets.
[3] Body weight gained divided by the protein intake for the 4-day period during the nitrogen balance studies.
[4] Standard error of the mean.

Nitrogen balance studies

Nitrogen balance determinations were made on the series of animals presented in table 2 for a 4-day period between the 4th and 5th weeks of experimentation, using the method described by Wissler et al. ('48) slightly modified. This period was considered adequate since the animals had been on their diets for 4 weeks and should have been in nitrogen equilibrium. All the animals on the experimental rice diets with and without antibiotics retained essentially the same amount of nitrogen per rat per 4-day period. The average protein intake for each group, except for those on terramycin, was the same, even though the animals were allowed food and water ad libitum (table 3). No doubt the differences in nitrogen balance for this period were not of sufficient magnitude to be detected by the method used. It is interesting to note, however, that protein efficiency (gain in weight: protein consumed) was improved with aureomycin and penicillin. The protein efficiency values reported here are in agreement with the values obtained in earlier experiments based on a two- or three-week period. These observations show that carcass weight and body length had definitely been increased by the addition of antibiotics, but give no indication as to actual body composition. A more detailed report on nitrogen metabolism and carcass composition will be presented at a later date.

SUMMARY

Penicillin and aureomycin added to a lysine-threonine-supplemented rice diet with a nitrogen content equivalent to 6.17% protein produced a growth response in weanling rats approximately equal to the growth obtained with diets containing 15% casein. Penicillin generally gave the greater growth stimulation. Chloromycetin, bacitracin, streptomycin, and terramycin had little or no effect in this diet. Most of the growth-stimulating effect of the antibiotics occurred during the first 10 weeks after weaning. A basal 90% rice diet

without supplementary amino acids gave significantly greater growth when each of the above-mentioned antibiotics, except chloromycetin, was added singly. Vitamin B_{12} did not improve any of these diets except the lysine-threonine-supplemented rice diet plus aureomycin. However, vitamin B_{12} and urea added to the lysine-threonine-supplemented rice diet gave a growth response equivalent to that obtained with the antibiotic-supplemented rice-lysine-threonine diet. This response was not obtained in the absence of vitamin B_{12}.

The organ: body weight ratios were the same in animals with and without antibiotics, indicating that the antibiotics produced a general increase in growth.

The protein efficiency ratios of rats receiving the rice-lysine-threonine diet and an antibiotic were much better than the ratios of the animals receiving the same diet without an antibiotic.

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A QUANTITATIVE STUDY OF RUMEN SYNTHESIS IN THE BOVINE ON NATURAL AND PURIFIED RATIONS

III. RIBOFLAVIN, PANTOTHENIC ACID AND NIACIN [1]

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Experimental work with respect to the apparent synthesis of certain members of the vitamin B complex by the ruminant has been comprehensively reviewed by Kon and Porter ('47). Wegner et al. ('41) reported that as the nitrogen (urea) content in the ration of a calf increased, the riboflavin content of the rumen ingesta decreased, but the pantothenic acid and niacin contents were not influenced. Lardinois et al. ('44) showed that the addition of urea increased the amount of these B vitamins in the rumen ingesta, provided a soluble carbohydrate was present. From slaughter data, Kesler and Knodt ('51) found that the levels of riboflavin and niacin were generally higher in all parts of the digestive tract than were present in the feeds the calves had received. The riboflavin and niacin contents of the small intestine were greater than in other regions of the tract.

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[2] This article is part of a dissertation presented to the Faculty of the Graduate School of Michigan State College in partial fulfillment of requirements for the degree of Doctor of Philosophy.

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In general, the literature indicates only that appreciably larger amounts of the various B vitamins are present in the rumen or secreted in milk than are ingested in the ration, but no attempt has been made to make a quantitative evaluation of the amounts synthesized. The object of this paper is to report the amounts of riboflavin, pantothenic acid and niacin synthesized in the rumen of fistulated calves which were maintained on a purified ration in which urea supplied the only significant source of nitrogen and starch and glucose the only sources of energy. One rumen fistulated calf was maintained on a natural ration to obtain ingesta for comparative purposes.

EXPERIMENTAL

The data concerning the calves, the composition of the rations and the weights of rumen contents have been reported previously (Agrawala et al., '53; Duncan et al., '53). The calves were fed once and watered twice daily. On the days when samples were taken, the entire rumen contents were removed through a fistula before the calf was fed (zero-hour sample). The evacuated contents were weighed and thoroughly mixed and a 500-gm aliquot was saved for analysis. The remaining contents were replaced immediately in the rumen and the calf was fed. Except for feeding, the same procedure was repeated 6 hours later (6-hour sample). The B vitamin content of the purified ration was negligible, although trace amounts were present as contaminants of the starch and lard. The natural ration contained 4.9, 8.5 and 25 μg per gram of ration of riboflavin, pantothenic acid and niacin, respectively.

Riboflavin was determined by the method proposed by Snell and Strong ('39) after an extract had been prepared by autoclaving a 1-gm sample of the feed or dried rumen ingesta in 0.1 *N* HCl for 30 minutes at 15 lb. pressure, cooling to room temperature, centrifuging at 3,500 r.p.m. for 15 minutes and adjusting an aliquot to pH 4.6 with NaOH. The filtrates were placed in low actinic flasks and stored in the refrigerator

until analyzed. *Lactobacillus casei* (7469) was used as the test organism.

The method of Krehl et al. ('43) was used to determine niacin. The samples were prepared in the same manner as for riboflavin except that 1 *N* HCl was used for the extraction. *Lactobacillus plantarum* 17–5 (8014) was used as the test organism.

The method proposed by Skeggs and Wright ('44), with the modifications suggested by Buskirk et al. ('48), was used to determine pantothenic acid. A 1-gm sample was digested with 1 gm of Mylase-P, buffered to pH 4.2 to 4.5 with 10 ml of 1 *N* acetic acid and 1 ml of 1 *N* NaOH, and incubated for two to three hours at 50°C. The same test organism was used as for niacin.

Both the rumen and feed samples were extracted with ether before the microbiological determinations were made.

RESULTS AND DISCUSSION

The results obtained from the microbiological assay of riboflavin, pantothenic acid and niacin, both before and 6 hours after feeding, are expressed as micrograms per gram of dried rumen ingesta and presented in table 1. The amounts of the B vitamins in the rumen of the calf on the natural ration were remarkably uniform at the two sampling periods (trial 8). In contrast, the amounts of these vitamins in the ingesta of the calves on the purified ration showed large and non-uniform increases within 6 hours after feeding. The average amounts of riboflavin, pantothenic acid and niacin in the rumen of the experimental calves before feeding were 4.8, 9.5 and 38.0 μg/gm, and they were 9.0, 16.0 and 71.0 μg/gm, respectively, 6 hours after feeding. The amounts present in the ingesta of the calves on the purified ration 6 hours after feeding are in agreement with respect to the amounts of riboflavin and niacin in the ingesta of the calf on the natural ration, but are approximately 50% lower in pantothenic acid. The values obtained for pantothenic acid and niacin for the calf on the natural ration compare favorably with those re-

ported by Wegner et al. ('41), but the riboflavin value reported here is approximately 50% lower.

By removing the rumen contents of the calves, both before and after feeding, it was found that there was no measurable synthesis of B vitamins in the calf on the natural ration, whereas an appreciable synthesis had occurred from the ingestion of the purified ration (table 1).

TABLE 1

The amounts of B vitamins synthesized in the rumen within 6 hours after feeding

(Expressed as micrograms per gram of dry matter)

TRIAL NO.	CALF	TIME	RIBOFLAVIN	PANTOTHENIC ACID	NIACIN
		hr.			
1[1]	C711	0	3.9	11.6	23.1
		6	6.8	16.5	29.2
2	C714	0	4.3	5.3	29.0
		6	11.0	18.5	90.0
3	C714	0	6.6	8.5	57.0
		6	10.0	11.2	91.8
4	C714	0	5.0	11.2	32.0
		6	9.0	20.1	85.0
5	C707	0	5.0	5.3	77.0
		6	8.8	11.3	85.0
6	C714	0	3.8	11.6	15.0
		6	7.1	14.9	24.7
7	C714	0	4.8	13.3	33.0
		6	11.8	16.5	91.8
8[2]	C714	0	9.0	28.0	60.8
		6	9.0	27.0	60.0

[1] A B vitamin-free purified ration was fed in trials 1 to 7, inclusive.
[2] A natural ration was fed in trial 8.

The data in table 2 show the actual number of milligrams of the B vitamins present in the rumen of the calves before and after feeding. The higher values obtained after feeding demonstrate that the rumen microorganisms were able to synthesize large amounts of riboflavin, pantothenic acid and niacin from a purified ration in which urea, glucose and starch supplied the only essential sources of nitrogen and

carbohydrate. On a percentage basis, more riboflavin was synthesized on the purified ration than appeared to be synthesized from the natural ration. The total amounts present in the rumen after feeding were approximately the same on both rations. Actually, the amount of riboflavin in the rumen of the calf on the natural ration at 6 hours can be accounted for by the amount ingested in the ration. Hunt et al. ('43) showed that the amount of riboflavin in the rumen was 7 to

TABLE 2

The amounts of B vitamins synthesized in the rumen within 6 hours after feeding

(Expressed as milligrams of total rumen dry matter)

TRIAL NO.	TIME	RIBOFLAVIN	PANTOTHENIC ACID	NIACIN
	hr.			
1	0	10.2	30.3	60.4
	6	26.3	63.8	113.0
2	0	6.7	8.2	45.0
	6	32.3	54.4	264.6
3	0	10.7	13.7	92.1
	6	28.0	31.4	256.9
4	0	8.3	18.6	53.2
	6	25.3	56.4	238.7
5	0	9.8	10.4	151.4
	6	33.0	42.4	319.2
6	0	5.4	16.6	21.4
	6	20.4	42.8	70.9
7	0	9.6	26.5	66.3
	6	38.8	54.2	301.7
8	0	18.9	58.7	127.4
	6	31.6	94.8	210.7

10 times greater than that in semi-synthetic rations which contained varying amounts of carbohydrate.

An appreciable synthesis of pantothenic acid occurred when the purified ration was fed, but the average amount present in the rumen 6 hours after feeding was approximately 50% less than that obtained when the natural ration was fed. No explanation for the decrease is readily available, but several factors may be contributory: (a) sensitivity of pantothenic

acid to changes in pH; (b) some essential nutrient for the synthesis of pantothenic acid may have been the limiting factor, namely, β-alanine; or (c) rapid utilization or removal of pantothenic acid from the rumen.

There was less than one-half as much niacin in the rumen of the experimental calves before feeding as was present in the rumen of the calf on the natural ration. Six hours after feeding the purified ration, the concentration of niacin increased from two to 5 times that present before feeding, whereas the concentration was only doubled in the rumen of the calf on the natural ration. In 5 of 7 trials the increase in niacin exceeded that present in the rumen of the calf on the natural ration. On the basis of the results obtained, the rumen microorganisms were able to synthesize as much or more niacin from the components of the purified ration as from the natural ration.

The data obtained from the calf on the natural ration do not indicate any synthesis of B vitamins, because the amounts ingested in the ration accounted for the apparent increases recorded in table 2. This does not mean, however, that synthesis did not occur, but due to the complexity of dealing with a continually moving system, no method is available at the present time to study this phenomenon quantitatively.

Since the purified ration was essentially B vitamin-free, the increases obtained in riboflavin, pantothenic acid and niacin are assumed to be due to biosynthesis of the components of the purified ration by rumen microorganisms. The increases observed in the amounts of B vitamins in the rumen at the two specified times do not take into consideration the synergistic utilization or passage from the rumen. Since these vitamins are water-soluble, it is probable that considerable quantities were absorbed or passed from the rumen, in which case the amounts determined 6 hours after feeding would be considerably less than the total amounts synthesized.

SUMMARY

The ingesta obtained from 7 trials with rumen fistulated calves were analyzed microbiologically to determine the capacity of rumen microorganisms to synthesize riboflavin, pantothenic acid and niacin from the medium components of a purified ration which contained urea as the only essential source of nitrogen.

Quantitative evidence was obtained to indicate that the microorganisms were able to synthesize as much riboflavin and niacin, but only 50% as much pantothenic acid, within 6 hours after feeding as was found in the rumen of a calf maintained on a natural ration.

The average of 7 trials showed that the calves on the purified ration synthesized 20, 31 and 154 mg of riboflavin, pantothenic acid and niacin, respectively, within 6 hours after feeding. The increased amounts of these B vitamins found in the rumen of the calf on the natural ration were approximately equivalent to the amounts ingested in the feed.

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THE EFFECT OF COPROPHAGY ON THE EXCRETION OF B VITAMINS BY THE RABBIT [1]

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The observation that rabbits excrete two types of feces was first made by Morot (1882) and has since been confirmed by several workers (Madsen and Taylor, '39; Eden, '40a; Southern, '40). The familiar dry type of pellet is voided chiefly during the day. The soft or night feces are voided chiefly at night. The night feces are lighter in color and covered with a tough mucous membrane. They are rarely seen, since the rabbit collects them directly from the anus and consumes them. The coprophagy occurs even when the animals are kept on wire bottomed cages, but can be prevented by using a specially designed collar. The soft feces may constitute as much as one-third of the total feces.

The composition of the soft feces and hard feces has been studied by Eden ('40b). He found that the soft feces contained 28.5% protein as contrasted with 9.2% in the hard feces, whereas the fiber and nitrogen extracts were higher in the hard feces. It is apparent from these figures that coprophagy is a normal process and plays a definite role in the nutrition of the rabbit. On the basis of the dietary requirements of the rabbit for the B vitamins (Olcese, Pearson and Schweigert, '48), this species is more like polygastric than monogastric animals. It is possible that the B vitamins are synthesized by microorganisms chiefly in the cecum of the rabbit and are then absorbed mainly from the reingested feces. However, the extent of microbial activity in the rabbit

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seems to be of a lower magnitude than in polygastric animals, as urea nitrogen cannot be used to replace dietary protein (Olcese and Pearson, '48).

The present investigation was undertaken with the object of determining the distribution of several B vitamins between the soft and hard feces and the effect of collaring to prevent coprophagy on the fecal and urinary excretion of these vitamins. Information is also provided for the first time on the excretion of vitamin B_{12} by rabbits.

EXPERIMENTAL

New Zealand White rabbits of mixed sexes were used in these studies. The rabbits used for the coprophagy studies were fed a standard commercial pelleted feed. The rabbits used for the B_{12} balance studies were fed a diet having the following percentage composition: corn 19, modified cornstarch [2] 11, cerelose 14.5, soybean protein 25, raw sugar 10, peanut oil 5, wood pulp 7, agar 5, salts mixture 3, vitamin A and D oil 0.5. The following supplements were added per kilogram of diet: DL-methionine 7 gm, glycine 4 gm, choline chloride 2 gm, mixed tocopherols 500 mg, thiamine 7 mg, and 2 methyl, 1-4-naphthoquinone 0.75 mg. The animals were kept in individual metabolism cages with wire bottoms. The collection period was for three consecutive days. The collars for preventing coprophagy were constructed of plywood and were about 10 inches in diameter. The urine was collected in brown bottles with sufficient acid to maintain a pH below 6.8. The feces were dried prior to analysis so that all values are expressed on an air dry basis.

Niacin, riboflavin and pantothenic acid were determined on the feces and urine by the standard microbiological assays. *L. arabinosus* was used for the niacin and pantothenic acid assays, and *L. casei* for riboflavin. Vitamin B_{12} was determined by the method of Skeggs et al. ('50), with the modification that sodium cyanide was added to the medium.

[2] Amidex, Corn Products Refining Co., New York, N. Y.

RESULTS AND DISCUSSION

The average daily feed intake of 6 rabbits during the uncollared period was 134 gm, and for the collared period 137 gm. Therefore, it may be assumed that the difference in rates of excretion are due to the coprophagy rather than to the amount of feed consumed.

Of the total feces recovered during the uncollared period, approximately 95% were hard feces, while only 5% were soft or night feces. During the collared period the average daily excretion of hard feces was 53 gm and of soft feces 19 gm. During the period the rabbits were collared the average daily recovery of both hard and soft feces was 72 gm, as compared with 56 gm recovered during the uncollared period. This indicates that feed is much more efficiently utilized by the rabbit when coprophagy is permitted than when the animals are prevented from consuming the soft feces.

The extent of microbial synthesis and absorption of niacin, riboflavin, pantothenic acid and vitamin B_{12} is apparent from a comparison of the amounts of these vitamins per gram of hard and of soft feces. The levels of each vitamin in the hard feces were of the same order of magnitude, irrespective of whether or not the rabbits were collared. This was also true for the soft feces. The essential difference is that from rabbits while collared 29% more feces were recovered than when the animals were not collared. This difference is essentially accounted for by the amount of soft feces excreted by the collared rabbits.

It may be of some significance that the levels of each of the 4 vitamins in the hard feces were slightly higher during the collared period than during the uncollared period.

The levels of niacin and riboflavin were between three and 4 times greater in the soft feces than in the hard feces, while the level of pantothenic acid was 6-fold greater in the soft feces. The latter are between two and three times higher in vitamin B_{12} than are the hard feces. This difference in the levels of these 4 vitamins in the hard and soft feces shows that there is considerable synthesis of these vitamins in the

gastrointestinal tract of the rabbit, and affords an explanation of why riboflavin and pantothenic acid are not dietary essentials for this species.

While there is apparently considerable microbial synthesis of niacin in the rabbit, it is not sufficient for maximum rates of gain unless the diet also contains tryptophan over and above the growth requirements for conversion to niacin (Olcese, Pearson and Sparks, '49). There was no significant or consistent difference in the levels of vitamins in the urine of rabbits that were collared to prevent coprophagy and in the

TABLE 1

Niacin, riboflavin, pantothenic acid and vitamin B_{12} in feces and urine of rabbits

(values per gm or ml)

	HARD FECES	SOFT FECES	URINE
Niacin, uncollared, μg	39.7	139.1	3.5
Niacin, collared, μg	41.5	147.2	2.4
Riboflavin, uncollared, μg	9.4	30.2	6.2
Riboflavin, collared, μg	9.8	40.6	6.6
Pantothenic acid, uncollared, μg	8.4	51.6	5.7
Pantothenic acid, collared, μg	10.0	63.7	7.9
Vitamin B_{12}, uncollared, mμg	892	2,922	1.7
Vitamin B_{12}, collared, mμg	933	2,031	1.4

urine during the period the rabbits were allowed to ingest the soft feces.

The excretion of niacin, riboflavin, pantothenic acid and vitamin B_{12} can be calculated by multiplying the levels per gram of feces, as shown in table 1, by the average grams of feces excreted daily over the three-day collection period. When coprophagy was prevented, the amounts of each of the 4 vitamins recovered in the feces were significantly higher than during the period the rabbits were uncollared. The average daily fecal excretion of niacin by the rabbits while collared was 4,676 μg, as compared to 2,553 μg recovered during the uncollared period. The calculated amount of each

vitamin absorbed due to ingestion of the night feces is obtained by subtracting the amount excreted daily by the rabbits while uncollared from the amounts recovered while collared. This would mean that through coprophagy 2,123 μg more of niacin is absorbed daily, or that the uncollared rabbits have available 83% more niacin than they would if collared to prevent ingestion of the feces. Ingestion of the soft feces provides double the amount of riboflavin that would be available if coprophagy were prevented. The ingestion of the feces provided these rabbits with 165% more pantothenic acid and 42% more vitamin B_{12} than was available when copro-

TABLE 2

Effect of coprophagy on the fecal excretion of niacin, riboflavin, pantothenic acid and vitamin B_{12}

	UNCOLLARED	COLLARED	CALCULATED AMOUNT ABSORBED DUE TO COPROPHAGY
	μg/day	*μg/day*	*μg/day*
Niacin	2,553	4,676	2,123
Riboflavin	595	1,196	601
Pantothenic acid	619	1,641	1,022
Vitamin B_{12}	58	82	24

phagy was prevented. It is apparent from these figures that coprophagy or consumption of the soft or night feces plays an important role in the nutrition of the rabbit, and that this accounts for the fact that the dietary B vitamin requirements of this species are more like that of polygastric animals than of monogastric animals such as the rat or human.

Vitamin B_{12} balance

Nine weanling rabbits were fed a diet containing only 0.4 mμg of vitamin B_{12} per gram. The amounts of B_{12} excreted in the feces and urine, and also amounts in liver, kidney and spleen, were determined. A record was kept of the amount of feed consumed during the three-day collection periods. No attempt was made to prevent coprophagy. The feces con-

tained an average of 842 mμg of vitamin B_{12} per gram. The average daily excretion was 221-fold greater than the daily intake of vitamin B_{12}. The average daily urinary excretion of vitamin B_{12} was 580 mμg. Thus it is apparent that relatively large amounts of vitamin B_{12} are synthesized by microorganisms in the gastrointestinal tract of the rabbit. While these experiments do not demonstrate that vitamin B_{12} is not a dietary essential for the rabbit, on the basis of the extent of microbial synthesis it would be expected that its needs are normally met by this source.

The amounts of vitamin B_{12} per gram of fresh tissue were: kidney 730 mμg, liver 340 mμg, and spleen 90 mμg. These values are of the same order of magnitude but slightly lower than the 1,107 mμg for kidney, 444 mμg for liver and 155 mμg for spleen of the chick, as reported by Yacowitz et al. ('52).

SUMMARY

Rabbits that are collared to prevent coprophagy or consumption of the soft feces excrete approximately 29% more feces than are recovered when coprophagy is not prevented. The difference is essentially accounted for by the soft feces excreted. The soft feces contain between three and 4 times more niacin and riboflavin per gram than do the hard feces. Pantothenic acid is about 6 times greater in the soft feces and vitamin B_{12} between two and three times the level in the hard feces. On the basis of the difference in the fecal excretion of the vitamins or their recovery in the feces of the rabbits while collared as compared with the amounts recovered while the animals were not collared, it can be calculated that coprophagy provides the rabbit with about 83% more niacin, 100% more riboflavin, 165% more pantothenic acid and 42% more vitamin B_{12} than would be available if the soft feces were not consumed.

The amount of vitamin B_{12} recovered in the feces of rabbits fed a diet low in this vitamin was 221-fold greater than the intake. Values are given for the vitamin B_{12} content of the liver, kidney and spleen of the rabbit.

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MICROORGANISMS IN THE INTESTINES OF RATS FED PENICILLIN[1]

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The addition of an antibiotic to the diet of a non-ruminant frequently results in an increased rate of growth. In the rat this increase is particularly great when the diet is limiting in a B vitamin (Linkswiler et al., '51; Lih and Baumann, '51; Sauberlich, '52), while the same antibiotics fail to stimulate growth when added to a complete ration. Tentative explanations of the growth-promoting effect are that the antibiotic may suppress adverse ("disease-producing") organisms, that it may suppress organisms which divert limiting nutrients from the host either by destruction or by incorporation into the bacterial cell ("vitamin-stealing"), or that it may stimulate the development of microorganisms which synthesize the missing vitamin. Intestinal microorganisms are emphasized, because the only known property common to the antibiotics as a group is an effect on microorganisms, and because analogous effects of sulfa drugs have also been ascribed to changes in intestinal flora (Daft and Sebrell, '45; Elvehjem, '48). This emphasis has been greatly strengthened by the announcement that antibiotics have thus far failed to stimulate the growth of germ-free chicks (Luckey, '52).

The purpose of the present study was to determine whether the numbers of certain intestinal organisms of rats are altered

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by the low concentrations of penicillin that stimulate the growth of the rat. To date such studies have been made on chickens (Elam et al., '51a, b; March and Biely, '52; Seiburth et al., '51; Rosenberg et al., '52), which, however, differ from rats in that their response to antibiotics seems to be much less dependent on the completeness of the diet (Stokstad and Jukes, '49, '51; Monson et al., '52). Johansson et al. ('52) have reported that high levels of aureomycin reduce the numbers of *Lactobacillus* spp. and *Clostridium* spp. in the intestines of rats, and increase the numbers of aureomycin-resistant coliforms.

EXPERIMENTAL

Weanling rats [3] weighing 38 to 50 gm were placed individually in wire-bottom cages. The basal diet had the following percentage composition: sucrose 73, vitamin-free casein [4] 18, salts [5] 4, corn oil 5, with the following vitamins added, in milligrams per kilogram of diet: thiamine hydrochloride 2.0, riboflavin 3.0, pyridoxine hydrochloride 2.0, nicotinic acid 25.0, calcium pantothenate 20.0, menadione 10.0, biotin 0.1, folic acid 2.0, vitamin B_{12} 0.02, inositol 100.0, choline chloride 1,000, α-tocopherol 30.0; 200 mg of halibut liver oil were added per kilogram of diet as a source of vitamins A and D. A "low-thiamine" modification of this ration contained only 1.0 mg of the vitamin per kilogram, chosen to produce approximately half-maximum growth. Penicillin G Na [6] was added to one-half of each diet at a level of 25 mg per kilogram. Food and water were given ad libitum and the animals were weighed at weekly intervals or when killed. The experiments were continued for 4 weeks.

The experiments were run in series of 20 to 30 rats each; half of the rats in each series received penicillin. A sufficient number of rats were killed at intervals so that pooled samples could be taken for bacteriological analysis. In a typical

[3] From the Holtzman-Rolfsmeyer Co.
[4] Labco.
[5] Wesson.
[6] Merck.

series 4 animals were killed after one day on the control diet, three from each group were killed after 7 and 9 days, and two after 14, 16 and 28 days, respectively. The rats were chloroformed, the abdomen was opened under aseptic conditions, and the intestinal tract removed to a sterile petri dish. The small intestine was divided into the upper and lower halves and the cecal pouch was dissected out. The contents of these parts were squeezed out with an alcohol-flamed smooth forceps, and the material from two to 4 animals pooled. A 0.5 to 1.0-gm portion was then weighed to the nearest milligram and transferred to a bottle, with 49.5 ml of sterile tap water, containing a half-inch layer of glass beads. This original suspension was shaken vigorously and then serially diluted by steps of 100, three times in the case of the two halves of the small intestine, and 4 times for the cecum contents. Most of each sample was used for determination of dry weight at 100 to 110°C.

The types of microorganisms measured were anaerobes (plate count under anaerobic conditions), coliforms, lactobacilli, and enterococci. They were determined by the following procedures:

Anaerobes: Pour plate method, with fluid thyoglycollate medium (BBL) hardened by adding agar to 1.5%. Counts were made after 120 hours of incubation at 37°C. under anaerobic conditions (oat-jar method).

Coliforms: Pour plate method, with eosin-methylene blue agar [7] after 72 hours of incubation at 37°C.

Lactobacilli: Agar shake tube method with carrot liver extract agar (Garey et al., '41) after 72 hours of incubation at 37°C.

Enterococci: Most probable number on the SF broth of Hajna and Perry ('43) [8] after 72 hours of incubation at 45°C.

The plates and shake tubes were all prepared in duplicate. The SF broth cultures for enterococci were prepared by using

[7] Difco.
[8] See footnote 7.

5 tubes per dilution. Colony counts were made of suitable agar plates and shake tubes with the aid of a Quebec colony counter. The most probable numbers of enterococci as shown by SF broth cultures were calculated from an appropriate table (Hoskins, '34).

All results were expressed as numbers of bacteria per gram of dry weight of the samples, since the presence of penicillin in the diet did not exert any consistent effect on the percentage dry weight of the intestinal contents.

RESULTS

The growth of the animals on the complete diet was good, without evidence of improvement due to the presence of penicillin. Since the animals were killed at intervals, not enough were alive at the end of the experiment for exact conclusions on growth to be drawn. Others, however, have consistently failed to observe any growth stimulation due to penicillin fed in similar complete diets (Lih and Baumann, '51; Sauberlich, '52). On the diet low in thiamine, the presence of penicillin was accompanied by a marked increase in growth (table 1), in agreement with previous observations.

TABLE 1

Effect of penicillin on the growth of thiamine-deficient rats

PENICILLIN	SERIES	GRAMS GAINED IN	
		14 days	28 days
0	III	46 (37–51)	57 (41– 60)
25 mg/kg	III	54 (46–67)	120 (98–131)
0	IV	45 (42–46)	58 (46– 65)
25 mg/kg	IV	57 (54–66)	115 (96–125)

The results of the bacteriological analyses are summarized in tables 2 and 3.

In spite of rather marked fluctuations in the numbers of organisms within any group of animals, certain changes appeared to be associated with the presence of penicillin in the

TABLE 2

Effect of penicillin on numbers of microorganisms per gram of dry intestinal contents of rats on a complete synthetic diet

DAYS	ANAEROBES		COLIFORMS		LACTOBACILLI		ENTEROCOCCI	
	No penic.	+ penic.	No penic.	+ penic.	No penic.	+ penic.	No penic.	+ penic.
				Upper half of small intestine				
0	17×10^7		11×10^5		3×10^5		1×10^3	
7	70×10^7	20×10^5	56×10^7		19×10^5	29×10^5	19×10^5	6×10^4
9	83×10^7	5×10^6	7×10^5	27×10^5	16×10^5	10×10^6	2×10^5	16×10^4
14	69×10^7	40×10^6	12×10^5	48×10^7	4×10^7	5×10^6	2×10^5	4×10^4
16	80×10^7	21×10^7	11×10^5	49×10^7	19×10^5	20×10^6	23×10^5	30×10^4
28	2×10^7	10×10^5	2×10^5	12×10^5	2×10^7	25×10^7	2×10^4	12×10^4
				Lower half of small intestine				
0	17×10^9		47×10^8				1×10^4	
7	17×10^9	13×10^5	3×10^7	83×10^7	17×10^9		58×10^6	4×10^6
9	7×10^8	5×10^7	1×10^7	3×10^7	40×10^9	22×10^7	6×10^6	58×10^6
14	5×10^8	14×10^9	48×10^7	24×10^9	1×10^9		29×10^6	28×10^6
16	8×10^8	6×10^8	2×10^7	35×10^8	12×10^9	77×10^8	10×10^6	19×10^6
28	1×10^8	5×10^7	4×10^5	5×10^7	2×10^9	2×10^7	3×10^4	5×10^4
				Cecum				
0	41×10^9		12×10^9		13×10^{10}		2×10^6	
7	47×10^9	49×10^8	75×10^7	19×10^9	17×10^{10}		14×10^6	88×10^6
9	36×10^9	3×10^8	86×10^7	3×10^9	66×10^9	40×10^{10}	16×10^6	10×10^6
14	63×10^{10}	10×10^9	13×10^7	12×10^9	42×10^{11}		2×10^6	79×10^6
16	81×10^9	51×10^9	30×10^8	45×10^9	15×10^{10}	40×10^9	33×10^6	3×10^6
28	2×10^9	9×10^8	5×10^6	1.4×10^9	2×10^9	28×10^9	3×10^6	28×10^6

TABLE 3

Effect of penicillin on numbers of microorganisms per gram of dry intestinal contents of rats on a low-thiamine ration

DAYS	SERIES	ANAEROBES		COLIFORMS		LACTOBACILLI		ENTEROCOCCI	
		No penic.	+ penic.	No penic.	+ penic.	No penic.	+ penic.	No penic.	+ penic.
				Upper half of small intestine					
0	IV	38×10^6		93×10^5		24×10^6		32×10^4	
7	IV	56×10^7	6×10^6	95×10^5	60×10^5	98×10^7	75×10^6	53×10^4	12×10^4
	III	50×10^7	29×10^6	12×10^6	28×10^6	89×10^7	18×10^6	11×10^4	34×10^4
14	IV	95×10^6	34×10^6	8×10^5	44×10^5	18×10^6		29×10^3	11×10^4
	III	94×10^6	6×10^6	44×10^5	67×10^5		4×10^6	1×10^3	38×10^4
28	IV	33×10^6	3×10^6	2×10^5	17×10^6			65×10^4	19×10^3
	III	9×10^6	97×10^6	4×10^5	14×10^6			2×10^3	1×10^3
				Lower half of small intestine					
0	IV	25×10^5		11×10^5		32×10^5		74×10^7	
7	IV	15×10^9	2×10^5	55×10^7	71×10^7	94×10^5	64×10^5	27×10^4	57×10^4
	III	94×10^5	2×10^5	14×10^7	15×10^5	12×10^9	44×10^5	27×10^4	63×10^4
14	IV	11×10^5	9×10^5	4×10^7	4×10^5	65×10^5	4×10^5	25×10^4	26×10^4
	III	21×10^9	53×10^5	4×10^7	68×10^5	56×10^5	57×10^5	28×10^5	19×10^5
28	IV	10×10^5	8×10^5	4×10^7	23×10^5			11×10^6	48×10^4
	III	4×10^5	10×10^5	1×10^7	10×10^5			4×10^4	5×10^4
				Cecum					
0	IV	61×10^5		16×10^5		19×10^{20}		33×10^4	
7	IV	37×10^9	25×10^5	86×10^7	59×10^5	32×10^9	42×10^9	9×10^4	15×10^5
	III	28×10^9	24×10^5	36×10^7	31×10^5	81×10^9		1×10^4	15×10^5
14	IV	12×10^9	14×10^9	77×10^7	50×10^5	18×10^9	60×10^9	39×10^4	4×10^5
	III	77×10^5	17×10^9	20×10^7	17×10^9	3×10^9	50×10^9	50×10^3	21×10^5
28	IV	36×10^5	45×10^5	3×10^7	60×10^5			41×10^3	36×10^4
	III	9×10^5	11×10^9	23×10^7	14×10^9			3×10^4	1×10^4

diet. During the first two weeks, the numbers of anaerobes were usually lower in the intestines and ceca of the rats fed penicillin than in those of the controls (table 2). In the absence of penicillin there appeared to be a moderate increase in anaerobes in the upper part of the small intestine for the first two weeks, and a marked decrease in coliforms in the lower part of the small intestine and in the cecum. Both latter effects could have been caused by the change from the preweaning diet of milk and crude stock ration to the purified experimental ration. In the rats receiving penicillin there was a marked increase in the numbers of coliforms in the cecum and in both parts of the small intestine (table 2). This effect of penicillin was evident throughout the experiment, the number of organisms being about 100 times as great in the presence of penicillin as in its absence.

The results for the lactobacilli were not considered reliable, since the relatively numerous coliforms produced gas in the tubes used for counting lactobacilli. Thus, some of the colonies observed might have been coliforms rather than lactobacilli. This inaccuracy was particularly serious in samples from rats fed penicillin. Nevertheless, certain counts were made in the highest dilutions in which no gas was produced. There appeared to be a slight diminution in the concentration of lactobacilli in the intestines of rats receiving penicillin (table 2), in spite of the "coliform effect" which would tend to make the values high. No consistent differences attributable to penicillin were noted in the concentrations of enterococci in the various parts of the intestinal tract.

The results obtained from rats on the "low-thiamine" rations (table 3) were qualitatively similar to those from animals on the complete ration (table 2). The increase in the concentration of coliforms in the groups receiving penicillin was definite at the end of 7 days and persisted through the 28th day. The numbers of anaerobes declined during the first week in the intestinal contents of animals receiving the antibiotic, but the effect disappeared after two weeks. There

were no consistent differences in the concentration of microorganisms attributable to the level of vitamin B_1 in the diet, with the possible exception of the numbers of anaerobes, which were somewhat higher in the cecum of the rats on the complete diet.

DISCUSSION

The numbers and kinds of organisms in the digestive tract may vary widely between individuals on the same diet or between groups of individuals examined at different intervals of time. This variability has been noted in both chicks and rats, on crude and synthetic diets (Nath et al., '48; Johansson et al., '48; Rosenberg et al., '52), and in the presence or absence of sulfa drugs (Gant et al., '43; Miller, '45; Evenson et al., '46), and also in chicks fed antibiotics (Elam et al., '51a, b; Rosenberg et al., '52). The present data show a similar variability. Nevertheless, it is apparent from tables 2 and 3 that the low levels of Na penicillinate fed produced changes in the intestinal flora and that these changes were in two directions; the anaerobes decreased, while the coliforms increased.

Changes in the upper part of the small intestine would seem to be particularly important from the nutritional point of view, since the organisms there would be favorably situated to destroy or absorb the limiting nutrient (thiamine, in the present experiment). If the chief function of the antibiotic were to favor thiamine synthesis, the change in organisms would also have to occur sufficiently high in the digestive tract so that absorption could still take place after the bacterial cells had disintegrated.

This leads to the suggestion that antibiotics may also stimulate growth by decreasing the survival time of certain of the bacterial cells. The number of organisms found in any area of the digestive tract might be considered the resultant of forces that favor the multiplication of bacterial cells and those that tend to destroy them; the total count does not indicate how long any particular type of cell survives. Others

have reported increases in antibiotic-resistant strains in the intestines of chicks (Elam et al., '51a, b) or rats (Johansson et al., '52) fed antibiotics, and these increases are presumably accompanied by decreases in other organisms. If the survival time of the individual cells decreased in a moving system such as the contents of the digestive tract, the numbers of cells dying in the small intestine would be increased, and the portion of bacterial vitamin available to the host would also be increased. Thus the host would be benefitted without there being an actual increase in thiamine synthesis. This point of view would be strengthened, if weaknesses — e.g., toward enzymes, bacteriophage, or other lytic agents — could be demonstrated in the intestinal bacteria from rats fed antibiotics.

SUMMARY

Rats were fed penicillin G sodium in a complete synthetic ration or in one low in thiamine, and the numbers of organisms in different parts of the intestinal tract were determined at intervals. Penicillin caused decreases in the concentration of anaerobes in the cecum and in both upper and lower small intestine during the first week of the experiment, and more persistent increases in the numbers of coliforms. The numbers of enterococci or lactobacilli were not altered by the levels of penicillin fed. The level of thiamine in the diet did not appear to affect the numbers of the various types of organisms studied.

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THE EFFECT OF ANTIBIOTICS AND THE COMPOSITION OF DIETS ON FECAL VITAMIN B_{12} AND BIOTIN [1]

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In a previous communication (Davis and Chow, '51) it was reported that supplementation of a basal soybean diet with aureomycin resulted in an increase in the vitamin B_{12} content of the feces. This effect was easily demonstrated, since the diet contained radioactive Co^{60}, which formed part of the vitamin B^*_{12} molecule.[2] The presence of radioactivity in the vitamin facilitates its identification and measurement by means of tracer techniques.

Increase in excreted vitamin is dependent on the amounts of antibiotic and Co^{60} used. The results of an extended study to ascertain whether other antibiotics or antibacterial agents have similar effects on the synthesis of vitamin B_{12} and one other vitamin, biotin, by the intestinal flora, and to evaluate the importance of proteins in the basal diets to which these substances were added, are reported in this communication.

EXPERIMENTAL PROCEDURE

Three experimental diets were used in this study. They contained 4.5% corn oil and 4% salt mixture IV (Hegsted

[1] The authors acknowledge grants-in-aid from the National Vitamin Foundation, Merck and Company, Inc., Sharp and Dohme, Inc., and the Upjohn Company.

[2] B^*_{12} is used here to designate radioactive vitamin B_{12}; B_{12}, the nonradioactive form.

et al., '41) and the usual vitamin supplement (Davis and Chow, '51), but differed from one another in the proportions of protein and sucrose which formed the remainder of the diets. Thus, besides fat and carbohydrate, the basal soybean meal diet (BSD) contained 62% soybean meal, and 29.5% sucrose; the basal casein diet (BCD) contained 22% crude casein and 69.5% sucrose; the protein free diet (PFD) contained 89.5% sucrose only. The protein contents (N × 6.25) of BSD and BCD were 25% and 22%, respectively, as determined by macro-Kjeldahl analyses. In the present experiments healthy adult rats of both sexes raised on stock diets (Barrows et al., '52) in our colony were employed. They were kept in individual metabolism cages and their dietary intake was limited to 12 gm per rat per day during the entire period of the study. The rats were allowed one week to acclimate themselves to the diets. On the 8th day, 1 ml of $CoCl_2$ solution [3] containing 20,000 counts per minute, equivalent to 0.2 μg of radioactive Co^{60}, was measured into every portion of the weighed diets. Urine specimens were discarded. Feces were collected twice daily and stored in a freezing cabinet; two days' specimens were pooled for analyses.

Each sample to be analyzed was homogenized with 80 ml of cold water and made up to 100 ml. A 10-ml aliquot was centrifuged in the cold and the clear supernatant fluid was transferred into a glass-stoppered graduated cylinder. One milliliter of B_{12} solution (50 mμg per milliliter) as carrier and 10 ml of aqueous saturated ammonium sulfate solution were added and the solution extracted with 30 ml of normal butanol previously saturated with water. After vigorous shaking, the content of the cylinder was allowed to stand at room temperature overnight. Twenty milliliters of the clear butanol extract were removed with a capillary into a 50-ml beaker. After evaporation of the organic solvent on a steam bath, the gummy residue was transferred onto a planchet with the aid of 95% ethanol and the radioactivity was meas-

[3] The $Co^{60}Cl_2$ solution with specific activity of 40 μc per milligram was obtained from Oak Ridge with the permission of the Atomic Energy Commission.

ured with a Geiger-Muller counter. Only one extraction was performed, since three additional ones with the same amounts of butanol yielded less than 15% of additional B^*_{12}.

RESULTS

The data in table 1 indicate that the increases in the vitamin B^*_{12} in the feces varied, depending on the antibiotic used for supplementation. Procaine penicillin (group C) showed an effect consistently and significantly above that shown by the controls (group G) in every period; aureomycin (group A) effects were equally consistent but much greater. The increases due to streptomycin or bacitracin were even greater than those seen with aureomycin during the first or second period but declined rapidly, despite continued administration of antibiotics and Co^{60}. After the third period with streptomycin and bacitracin, the excreted radioactivity was lower than that of the penicillin group and only insignificantly above that of the control group. The effect of neomycin [4] was marked but not sustained.

In a second similar experiment, sulfasuxidine, an effective bacteriostatic agent for certain microorganisms in the intestinal tract, was incorporated in the soybean diet at 0.10% and 0.50% levels, in place of the antibiotics. This compound produced a slight and temporary increase in the radioactive vitamin B_{12} content in the feces during the first two periods, but the vitamin content declined to the level of the controls in the 5th period. These results, taken as a whole, demonstrate that the ability of various antibiotics and a sulfa drug to stimulate an increase of fecal vitamin B_{12} is associated with their common antibacterial properties. However, the persistence of the effect upon prolonged feeding differs widely with the agent.

The effect of basal diet composition was also studied in a series of experiments in which aureomycin was added to BSD,

[4] Neomycin was kindly supplied by The Heyden Chemical Corporation, New York, N. Y. Bacitracin was obtained from the Commercial Solvents Corporation.

BCD and PFD rations. The same amount of Co^{60} was added to all diets. The results (table 2) demonstrate that under these conditions basal diet composition plays a significant role in the synthesis and fecal excretion of B^*_{12}. With PFD there was a marked but temporary increase, while both BCD and BSD gave more striking effects in both amount and duration of increased output. The significance of protein quality is suggested by the differences between BCD and BSD. These differences varied considerably in magnitude in several experiments. These data, taken collectively, therefore suggest

TABLE 1

The effect of various antibiotics on the fecal content of B_{12}[1]

Diet — Basal soybean diet (BSD)

SUPPLEMENT	PERIOD			
	1	2	3	4
A. Aureomycin 0.05%	300 ± 30	250 ± 18	350 ± 17	250 ± 22
B. Streptomycin 0.05%	450 ± 28	150 ± 10	80 ± 11	85 ± 10
C. Procaine penicillin 0.05%	100 ± 12	100 ± 14	100 ± 21	120 ± 11
D. Bacitracin 0.01%	450 ± 50	400 ± 28	100 ± 10	60 ± 8
E. Neomycin 0.05%	300 ± 38	550 ± 59	200 ± 18	100 ± 10
F. Neomycin 0.01%	50 ± 10	300 ± 38	110 ± 17	60 ± 10
G. None	40 ± 11	40 ± 8	60 ± 7	45 ± 17

[1] The average radioactivity is expressed in counts per minute ± standard error of the mean.

the importance of protein in diets in the manifestation of these antibiotic effects.

The quantity and quality of dietary proteins have marked effects on the fecal content of vitamin B_{12}, probably due to their influence on intestinal flora. Similar effects on urinary and fecal biotin excretion were observed when three groups of male adult rats, housed in individual metabolism cages, were offered PFD, BCD and BSD rations, respectively. Throughout the entire experiment all rats received the regular vitamin supplement, excluding biotin. Furthermore, alcohol-extracted vitamin-free casein was used in place of crude

casein in BCD. Daily collections of urine and feces were made. Subsequent microbiological assays (Wright and Skeggs, '44) for free biotin (table 3), employing *Lactobacillus arabinosus* 17-5 as the test organism, confirmed previous findings (Davis and Chow, '50) that the animals receiving dietary protein had significantly higher average free biotin levels in both urine and feces than the protein-free diet group. These values represent the data from 4 collection periods of 4 days each; the variations from samples are given by the standard deviations of the mean. It may be observed that there was only a slight difference in the excretion patterns of the two groups receiving dietary proteins.

TABLE 2

The effect of various diets on B_{12} [1] content in feces*

(Each basal diet contained 0.05% aureomycin)

BASAL DIET	PERIOD			
	A	B	C	D
Protein-free	100	350	320	200
Casein	150	720	1,000	1,000
Soybean	150	350	500	500

[1] Tabulated figures are for the average radioactivity in counts per minute.

Antibiotics are selective in their action on microorganisms. If antibiotics in the amounts necessary to produce changes in the fecal vitamin content actually alter the intestinal flora, with an accompanying change in capacity for the synthesis of various vitamins, it is conceivable that a given antibiotic may result in an increase in the synthesis of one vitamin and, simultaneously, a decrease in the synthesis of another. Experiments to test such a hypothesis were carried out by determining the free biotin content in the urine and feces of rats fed BCD supplemented with different antibiotics.

The experimental procedure of one of the typical experiments was precisely the same as that used for measurement of fecal B_{12}. Free biotin in urine was measured without hydrolysis. Fecal samples were homogenized and centrifuged

and the supernatant fluids were assayed for free biotin as described previously. Control recovery experiments demonstrated that the presence of antibiotics in the quantities existing in the diluted samples used for microbial assay did not in any way interfere with the analysis. The data (table 4) demonstrated that the fecal content of free biotin in the non-supplemented control group was relatively constant during the 4 experimental periods, totaling 12 days. The addition of antibacterial agents caused a decrease in two instances (aureomycin and streptomycin groups), no change in one (sulfasuxidine at both levels), and an increase in two (penicillin and bacitracin). These changes appear to have no rela-

TABLE 3

Effect of protein on urinary and fecal free biotin

DIET [1]	FREE BIOTIN, μG/SAMPLE	
	Urine	Feces
Protein-free	0.16 ± 0.01	0.05 ± 0.01
Casein	0.57 ± 0.02	0.40 ± 0.02
Soybean	0.51 ± 0.02	0.40 ± 0.02

[1] Biotin-free vitamin supplement.

tion to the effects of these agents on the fecal excretion of vitamin B_{12} reported in tables 1 and 2. For example, aureomycin, which effectively brought about an increase in B^*_{12}, resulted in a decrease in free biotin. On the other hand, procaine penicillin, which enhanced the production of biotin markedly, stimulated only a slight but sustained increase of vitamin B_{12}. Bacitracin increased the production of both biotin and vitamin B^*_{12}.

DISCUSSION AND CONCLUSION

Data are presented to demonstrate that the fecal excretion of vitamin B^*_{12} and free biotin is altered either by addition of antibacterial agents or by changes in the proteins in the basal rations, or both. Since each of the agents studied has

a different spectrum of activity, it is quite likely that any one agent may inhibit the growth of one species of organism and yet be without effect on another microorganism. Thus, depending on the ability of the surviving organisms to produce vitamins, and on their requirements, the net amount of the various vitamins in feces cannot be predicted. This is borne out by these experiments comparing vitamin B_{12} and free biotin.

It is now well established (Jukes et al., '50; Whitehill et al., '50; Catron et al., '51; Speer et al., '51) that the addition of antibiotics to animal feed increases not only growth rate but also feed efficiency. Most of the reports in this field deal with the effects rather than the mechanism of action

TABLE 4

The effect of various antibacterial agents on fecal content of free biotin

(Free biotin in μg/ml of fecal homogenate)

GROUP	SUPPLEMENT	%	INITIAL PERIOD	FINAL PERIOD
A	.....	0.0	1.9	1.8
B	Aureomycin	0.05	2.1	1.1
C	Streptomycin	0.05	1.9	1.5
D	Sulfasuxidine	0.10	1.7	2.0
E	Sulfasuxidine	0.50	1.5	1.8
F	Procaine penicillin	0.50	2.2	3.7
G	Bacitracin	0.01	1.5	2.8

(Atkinson and Couch, '51; Branion and Hill, '51), which is attributed to the modification of intestinal flora resulting in: (a) a disease control effect or (b) a nutritional effect, or both (Sieburth et al., '51). The present investigation offers direct and unequivocal evidence of an increase in B_{12} synthesis in the gastrointestinal tract when certain antibiotics are fed. However, there is as yet no conclusive experimental evidence (Schweigert et al., '45; Stokstad and Jukes, '50; Waisman et al., '51; McGinnis et al., '50) to show that increased synthesis of vitamins by the microorganisms in the large intestine necessarily implies increased absorption by the host.

If effective absorption does take place as a general phenomenon, it would be difficult to explain why pernicious anemia patients whose feces contain an abundance of B_{12} require an exogenous supply, either parenterally or orally adiministered (Girdwood, '50; West, '48). Furthermore, if absorption of vitamins produced by the microorganisms in the intestinal tract occurred, certain organs (Harte et al., '52; Rosenblum et al., '52; Chow et al., '51) of rats fed with radioactive Co^{60} and aureomycin should contain radioactive vitamin B_{12}. To ascertain whether they do, male and female rats were fed a soybean meal basal diet supplemented with Co^{60} and aureomycin for approximately 5 weeks. In the 6th and 7th weeks, Co^{60} was withdrawn from the diet, and three animals of either sex were necropsied in the 8th week. Livers, kidneys, pancreas, and so forth, were removed and assayed for radioactivity by wet ashing the entire organs. Each liver showed radioactivity equivalent to about 100 counts per minute; other organs had practically none. The radioactivity in liver can undoubtedly be due to Co^{60} (Comar et al., '46; Monroe et al., '52) as well as B_{12}. Because of the low radioactivity, no effort was made to differentiate these two compounds. These data therefore indicate that the absorption of the vitamin B^*_{12} synthesized in the intestinal tract was extremely poor. Had absorption been appreciable, retention of B^*_{12} in organs such as liver, pancreas, and so forth, for a matter of more than one month without appreciable loss of radioactivity should follow (Harte et al., '53).

However, it is also possible that the Co^{60}-containing compound soluble in butanol need not be vitamin B_{12}, and therefore there was a lack of radioactivity in the organs which possess a great affinity for radioactive vitamin B_{12}. Nevertheless, the belief that the radioactivity in the organic solvent is due primarily, though not completely, to B_{12}, is based on the following findings: (a) In experiments similar to those described in table 1, analyses for the microbiological activities in the butanol extracts showed a parallelism with the radioactivity; (b) chromatographic analyses of butanol ex-

tracts of feces from rats fed aureomycin and bacitracin showed the presence of a Co^{60}-containing fraction with R_f [5] essentially equal to that of radioactive vitamin tagged with Co^{60} as control.

It would be unwise to conclude from this study that, by analogy, all water-soluble vitamins produced by intestinal flora are poorly utilized. It must be pointed out that vitamin B_{12} (Chow et al., '50b; Yamamoto et al., '51; Chow et al., '50a; Conley et al., '51), unlike other water-soluble vitamins in the B group such as biotin (Oppel, '42; Najjar and Barrett, '45), thiamine or riboflavin (Mannering et al., '44) is poorly absorbed when administered orally. In contrast, it is of interest to note that there was an increase in biotin in the feces of rats fed a diet supplemented with antibiotics such as penicillin, and that the amount of free biotin activity in the urine of rats fed protein-containing diets was higher than that in the urine of animals on the protein-free diet. These data perhaps suggest an increase in the absorption of biotin paralleling the increase in synthesis by the microorganisms. The evidence would be more convincing if one were able to produce tagged biotin from the flora and to identify it in the urine.

In conclusion, the data reported at this time provide direct evidence of changes in fecal content of vitamin B^*_{12} and biotin due to feeding of antibacterial agents. The direction, magnitude and duration of the changes vary for each vitamin with the agents used. Whether the increase in the net production of vitamins in the large intestine results in absorption by the host still awaits further experimentation.

SUMMARY

Adult rats were fed a basal soybean diet to which were added as supplements a constant dose of $Co^{60}Cl_2$ and one of the several antibiotics or a sulfa drug. The fecal matter was collected, homogenized, and finally extracted with butanol.

[5] $R_f = \frac{\text{distance solute travels}}{\text{distance solvent travels}}$.

The radioactivity in the organic solvent was taken as an index of vitamin B_{12}. It was found that supplementation with certain antibacterial agents like aureomycin caused a marked and sustained rise in the butanol-soluble radioactive compounds. Some antibiotics produced a temporary but sharp increase and others only a slight but sustained rise. This effect of antibiotics was also dependent on the composition of the basal diet. Furthermore, it was found that one antibiotic may enhance the synthesis of vitamin B_{12} but may depress that of biotin, and vice versa. These results were interpreted in terms of the selective influence of antibiotics on the intestinal flora. Discussions of the absorption of nutrients synthesized in the large intestine are also presented.

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STORAGE AND ELIMINATION OF VITAMIN B_{12} IN THE RAT[1]

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ONE FIGURE

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Clinical experiences (West, '48; Conley, Green, Hartman and Krevans, '53) with vitamin B_{12} in the therapy of pernicious anemia indicate that prolonged remission follows the injection of single relatively large doses of the crystalline vitamin. This suggests the existence of some mechanism by which the vitamin is stored in one or more organs and more or less slowly released from these storage sites.

Previously, Rosenblum, Chow, Condon and Yamamoto ('53) showed that following subcutaneous injection of single 4.24-μg doses of radioactive vitamin B_{12}[2], activity was found in various organs and tissues (kidneys, muscle, liver, skin, testes, blood, heart, brain, spleen and marrow) of animals sacrificed 96 hours after dosing. These findings, along with others by Barbee and Johnson ('51) on the rat and by Monroe, Patrick, Comar and Goff ('52) on the chick, indicate the virtual ubiquity of B_{12} in the animal organism.

Availability of B^*_{12} (Chaiet, Rosenblum and Woodbury, '50) makes possible an extension of this study to include data on the rate of disappearance of the vitamin from selected organs and on the rate of turnover as it is related to continuing intake.

[1] Presented at the 16th annual meeting of the American Institute of Nutrition, April 15, 1952, in New York City.

[2] In what follows, vitamin B_{12} tagged with radioactive Co^{60} will be designated as "B^*_{12}"; crystalline non-radioactive vitamin will be written "B_{12}."

EXPERIMENTAL

Three experiments designed to yield information on the loci and duration of storage in certain tissues and organs, and on the mobility of vitamin stores, are reported on at this time.

The distribution and persistence of the parenterally-administered vitamin in certain organs

Thirty adult rats, equally divided as to sex, were housed in individual metabolism cages at the start of the experimental period and maintained on a stock diet containing 1% of liver. After one week on this regimen, urines were collected over a 24-hour period. At the end of this time each animal was given, by subcutaneous injection, 1 μg of B^*_{12}, with a specific activity of 40 μc per milligram. Following injection, urines were collected after the first and (except from the rats in the group sacrificed after the first day) second days. At intervals during 89 days, groups of 5 animals, mixed as to sex, were sacrificed. The cadavers were weighed, and the liver, kidneys, spleen, pancreas and reproductive organs (testes or uterus and ovaries) were dissected out and weighed. In addition, two 5-gm samples of muscle tissue were taken from the thighs.

Each urine sample was diluted to 200 ml with water. Fifty-milliliter aliquots were used for assay for vitamin B_{12} activity by the microbial adsorption technic of Davis and Chow ('52). The values so obtained were in some instances checked by the butanol extraction method following addition of B_{12} as a carrier.

For assay by the microbial adsorption technic, the aliquot of diluted urine was mixed with 1 ml of a thin suspension of *L. leichmannii*. After 15 minutes at room temperature the organisms were centrifuged down, and the supernatant fluid carefully removed with the aid of suction. The bacterial mass was quantitatively transferred to the planchet with the aid of water, evaporated to dryness and the cells counted.

In the butanol extraction technic, 50 μg of vitamin B_{12} and 20 gm of ammonium sulfate were added to the aliquot in a glass-stoppered cylinder. The aqueous solution was extracted 5 times with 20-ml portions of normal butanol saturated with water. The combined butanol extracts were evaporated to small volume, transferred to the planchet, further evaporated to dryness, and the cells counted.

Organ and tissue samples were prepared for assay by wet ashing with a mixture of nitric and sulfuric acids. To each sample 1 mg of cobaltous chloride was added as carrier. After evaporation of the acid the residue was transferred to the planchet and the radioactivity determined.

All counting was done with the aid of a Tracerlab Scaler 100, used in conjunction with a thin window counter.

None of the urine samples taken prior to administration of vitamin B^*_{12} had detectable radioactivity. During the 24 hours immediately following injection, the 30 animals excreted in their urine 77 ± 38 mμg [3] each. In the course of the second 24-hour period there was no detectable radioactivity in any of the samples from the 25 surviving animals. Assays by the microbial adsorption technic yielded uniformly higher values than those carried out by the butanol extraction procedure. In a series of 6 comparisons, results by the former method were $25 \pm 5\%$ higher. This would be expected for the results of recovery experiments where known amounts of vitamin B^*_{12} were added to normal rat urine. In the tests with the butanol extraction technic the recoveries were 75% and 85%, respectively, when 10 and 30 mμg were added; with the microbial adsorption technic, 92, 90, and 92% with additions of 5, 10, and 20 mμg.

In these experiments no attempt was made to control food intake. The question, therefore, arose of whether the amount of vitamin B^*_{12} found in an organ would be influenced by inanition. To effect partial exploration of this possibility, two groups of 4 females were injected with 1 μg of vitamin

[3] The standard deviation of the observations is used to indicate limits.

B^*_{12}, after which one group was fed ad libitum and the second allowed access to only half as much food. At the end of 48 hours, all animals were sacrificed. The findings, summarized in table 1, showed a mean weight loss of about 14% for the restricted group, a loss in liver weight of something more than 20%, but only a 4% reduction in vitamin B^*_{12} storage in the liver. The liver weight loss, according to Kosterlitz ('44) and Harrison and Long ('45), is largely in the glycogen, lipid, and water fractions under these conditions. Both the weight loss and the liver weight loss were significant; the reduction in stored vitamin was not significant. It appears, therefore, that under these conditions the proper criterion for the following comparisons is the measure of total vita-

TABLE 1

Effect of mild inanition of B^*_{12} in liver

(Four females in each group)

FEEDING	BODY WEIGHT	LIVER WEIGHT	RADIOACTIVITY IN LIVER
	gm	*gm*	*counts per min.*
Ad libitum	280	11.9	252
Restricted	237	9.4	241

min in the organs or tissues, rather than vitamin per gram of organ or tissue, since the latter may be generally more susceptible to the effects of variations in caloric intake. This is reasonable, too, since the dose administered was constant and independent of body weight.

In figure 1 radioactivity in selected organs is plotted as a function of time. Noteworthy are the initially large amounts found in the kidneys and the relatively rapid loss from this organ. Liver content of radioactivity similarly rises promptly to a moderate level, but subsequently there appears a levelling off. The pancreas, surprisingly, promptly stores a fraction of the injected dose, and the initial concentration attained did not change markedly over the three-month period of observation. The spleen retains only minimum amounts of activity,

which remain fairly constant. The observations on the gonads and muscle samples are not reported on in detail, since only very small amounts of radioactivity were found in these tissues.

Turnover of stored vitamin in stock ration-fed and saturated rats

A number of rats were given single subcutaneous injections of a mixture of equal parts of B^*_{12} and B_{12}, equivalent to

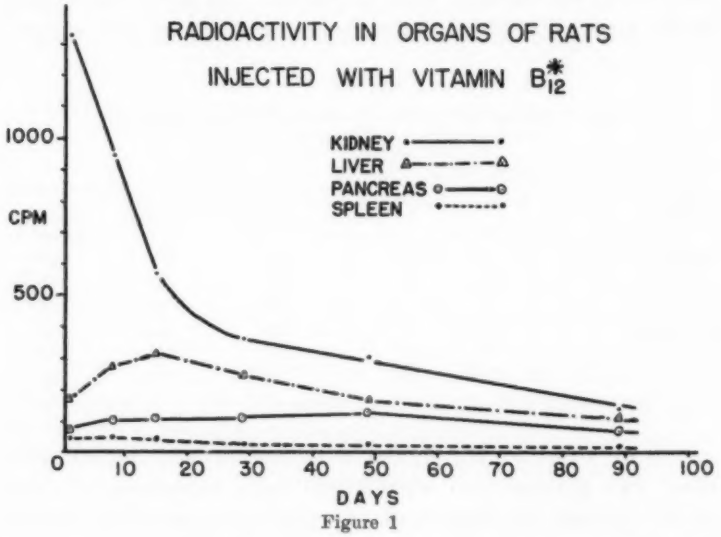

Figure 1

1 μg of B_{12} activity. These animals were maintained on the stock diet, and half of them were given daily subcutaneous injections of 50 μg of B_{12}. Animals from each group were sacrificed at various times. Radioactivity in various organs was determined as before. The data for liver, kidney and pancreas are shown in table 2.

The data fail to indicate any marked effect of continued massive injections of B_{12} on the stored B^*_{12} in the pancreas. In the liver the evidence is quite suggestive that stored radio-

activity is flushed out under these conditions, while the kidneys apparently retain radioactivity well, perhaps even better in the animals receiving B_{12} parenterally than in the controls.

These findings prompted an extension of the study of this aspect of the problem to animals saturated with B_{12}, and an evaluation of the role of continuing administration of excessive amounts of B_{12} on storage of B^*_{12} in already saturated animals. For this study three groups, each consisting of two males and two females, were employed. All were maintained on the stock diet, but two groups received daily oral supple-

TABLE 2

The "flushing out" effect of continued massive injections of B_{12} on the storage of B^*_{12}

GROUP	NO. OF RATS	DAYS	RADIOACTIVITY IN TARGET ORGANS (C.P.M.)		
			Liver	Kidney	Pancreas
Control	6	12–13	216	696	97
	4	25	227	224	78
	4	74–75	96	76	58
B_{12} injected	3	13	228	714	65
	3	21	126	835	67
	3	30	69	564	48

ments of 10 μg of B_{12} mixed with the ration. In addition, these two groups also were given daily injections of 50 μg of B_{12} during the two weeks of pretreatment given all the animals.

At the end of the two-week period, all animals were given a single injection of the mixture of B^*_{12} and B_{12}, equivalent to 1 μg of B_{12} activity. During the following 4 days, the control group and one of the "saturated" groups were maintained on the stock diet, while the second "saturated" group continued to receive additional B_{12} both orally and parenterally. During the first 48 hours after B^*_{12} injection, urines were collected from all animals. Four days after B^*_{12} injection, the animals were sacrificed and analyses for radio-

activity were carried out on the livers, kidneys and pancreases.

The data in table 3 indicate that: (1) the saturated animals excrete more B^*_{12} in the urine during the first 48 hours after injection than do the stock animals; (2) very large amounts of radioactivity concentrate in the kidneys; (3) less B^*_{12} is stored in the livers and pancreases of the saturated animals at the end of 4 days than in those of the controls; (4) strikingly more B^*_{12} is held in the kidneys of the saturated animals than in those of the controls; and (5) continuing administration of B_{12} to the already saturated animals has little

TABLE 3

Urinary excretion and target organ storage of radioactivity following B^*_{12} injection into rats variously treated with B_{12}

GROUP	TREATMENT WITH B_{12}		RADIOACTIVITY (C.P.M.)			
	Before	After	Urinary excretion (48 hours)	Liver	Kidney	Pancreas
A	—	—	67	172	536	74
B	+	—	160	79	1,754	26
C	+	+	180	95	1,795	30

or no effect on the storage of B^*_{12}. Whether these effects would persist, were the animals kept on the same regimens for longer periods, is presently unknown.

DISCUSSION

These experiments point out, for the first time, that significant and prolonged storage of B^*_{12} occurs in the pancreas of the rat. Whether this finding is meaningful in terms of the many well-recognized functions of this organ remains to be established. If B_{12} enters into any of the enzyme systems responsible for the synthesis of the digestive enzymes supplied by the cells of the pancreas, then a physiologic basis for the generally accepted stimulatory effect of the vitamin may be revealed by further search.

Little is known of the fundamental mechanisms involved in the synthesis of insulin. There is little doubt, however,

that insulin is an end product of some sequence of enzymic syntheses in the pancreas, and the possibility must be mentioned that B_{12} may function as a coenzyme in one or more of these reactions.

In certain tissues, at least, B_{12} is held tenaciously and evidently fails to equilibrate readily with additional supplies of the vitamin subsequently introduced into the organism. Whether this finding means that in certain sites B_{12} is irreversibly bound, remains a subject for future study.

Within the kidney and pancreas, at least, the amount of an administered dose retained is conditioned by the state of saturation of the animal. On the other hand, available evidence emphasizes the apparently unique role of the kidney in the metabolism of the vitamin. Within that organ extremely high concentrations are found, as well as marked shifts with time during prolonged periods of observation. Saturated animals apparently concentrate more of an injected tagged dose in their kidneys than do stock controls, under the experimental conditions employed in these studies, and this concentration is not modified by continued flooding of the host organism with the vitamin, at least during the relatively short periods studied.

The data on persistence of storage as determined in the rat may possibly offer a satisfactory explanation for the relatively prolonged therapeutic effects of B_{12} injections in pernicious anemia in man. To the extent that storage occurs, these data support the inclusion of B_{12} in the family of water-soluble vitamins. There is an analogy in that, for example, beri-beri or scurvy does not follow immediately on the heels of thiamine or ascorbic acid deprivation, but clinical response is dramatic when a vitamin is administered following the development of the symptoms of the disease.

It must be emphasized that the data presented in this report are not to be interpreted as quantitative, since no attempts were made to introduce all of the refinements requisite for the most precise determinations of radioactivity. Moreover, of necessity, the groups of animals employed

in these experiments were small in comparison with those commonly employed in the more conventional types of nutritional studies, because of the limited availability of B^*_{12}. While these findings are essentially qualitative, therefore, this fact has no influence on the validity of the broad conclusions drawn. As such, these broad conclusions are properly to be regarded as guideposts to future programs of study.

SUMMARY

Data are presented on the storage of radioactivity in selected target organs following administration to rats of Co^{60} containing vitamin B_{12}. Noteworthy is the persistence, up to three months, of significant storage in the pancreas.

The effects on storage in selected organs of flooding of the host with the vitamin have been studied. In this instance some evidence suggestive of flushing out in liver and pancreas has been found, but the storage of radioactivity in the kidney appears to be unaffected.

The state of saturation of the host has been shown to play a role in the extent of storage of a tagged dose in the liver, pancreas and kidneys. In the first two organs, storage is less in the saturated animal; in the kidneys, greater storage is seen.

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EVALUATION OF PROTEIN QUALITY IN COTTONSEED MEALS BY CHICK GROWTH AND BY A CHEMICAL INDEX METHOD

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Studies on the utilization of cottonseed meal protein by animals other than ruminants point to two major nutritional factors. The first is the necessity of reducing the content of free gossypol to fairly low levels in order to avoid the unfavorable physiological effects of this compound. Recent publications concerning this phase of the problem as related to chicks and laying hens include the reports by Boatner et al. ('48), Lillie and Bird ('50), Heywang et al. ('52b) and Heywang and Bird ('52a).

The second factor concerns the quality of the protein as modified by processing methods and other variables. Cottonseed is usually subjected to some degree of heat treatment during the commercial production of the meal. The detrimental effect of heating on cottonseed protein has been shown by Osborne and Mendel ('17), Gallup ('28) and Olcott and Fontaine ('41).

More recent reports indicate that low lysine availability is a major factor. Kuiken and Lyman ('48) and Kuiken ('52) showed that only part of the lysine in cottonseed meal was available for utilization by the rat and that lysine availability varied according to the processing conditions. A particularly significant finding was that lysine availability was

sharply reduced by heating cottonseed protein in the presence of both gossypol and cottonseed oil.[1] Heating the protein alone, with gossypol in the absence of oil or with oil in the absence of gossypol, had little effect on the availability of lysine. This points to gossypol as one of the factors in the reduction of lysine availability in cottonseed protein. It seems reasonable to postulate that the action of the oil was to dissolve the gossypol and thus bring it into contact with the protein. A lowered lysine availability which occurs when certain proteins are heated alone (Pader, Melnick and Oser, '48) appears to be due to a different type of reaction, since Kuiken found that autoclaving in the absence of gossypol had comparatively little effect on lysine availability in cottonseed.

The concept of bound gossypol proposed by Clark ('28) a number of years ago as an explanation of what happens when gossypol is inactivated during the processing of cottonseed meal, postulates that inactive gossypol is an insoluble gossypol-protein complex. The formation of an insoluble gossypol-protein complex which might be resistant to digestion could well account for substantial reduction in lysine availability and hence in lowered nutritional value of the protein. Gossypol protein complexes have been prepared in the laboratory by Hale and Lyman ('48) and by Castillon and Altschul ('50). The known reactivity of gossypol with amino groups suggests that the amino groups of lysine may be involved in the formation of gossypol protein complexes or bound gossypol.

Chick feeding tests with cottonsed meal showing a need for additional lysine have been reported by Sherwood and Couch ('50), and Richardson and Blaylock ('50a, b).

A fairly simple chemical test which would measure protein quality in samples of cottonseed meals would be of considerable value in processing tests for the improvement of this product. Olcott and Fontaine ('42) reported that cooking cottonseed proteins reduced the solubility in a dilute sodium

[1] Wesson.

chloride solution and proposed a protein solubility test as a means of determining when overcooking has occurred during the processing of cottonseed. Attempts in the authors' laboratory to use this test as a means of evaluating protein quality in cottonseed meals have been disappointing. Although a good correlation between the solubility data and the nutritional value of several limited series of cottonseed meals was found, quite a number of meals failed to show this relationship. Specifically, certain meals had extremely low protein solubility and yet had excellent protein quality as determined by both chick and rat feeding tests. Consequently, a more reliable way of evaluating protein quality in cottonseed meal by chemical tests has been sought. A good correlation between the enzymatic release of amino acids from cottonseed meal protein and chick growth rate was reported by Ingram et al. ('50).

In the present investigation, chick feeding tests in which all of the protein of the ration was supplied by cottonseed meal were used for the biological evaluation of protein quality. The same samples were assayed by a new chemical procedure and the results obtained by the two methods were compared.

EXPERIMENTAL

Chick feeding tests

The New Hampshire chicks used in these studies were obtained from hens maintained on litter and which had been fed a complete diet insofar as known nutrients and unidentified factors could be incorporated into the ration. The chicks were kept in batteries with raised screen floors, and feed and water were supplied ad libitum. The birds were weighed initially and at weekly intervals during the experimental period of 4 weeks.

The basal diet used in this study contained 4% of total fat, 5% salts IV, 40% cerelose and about 50% cottonseed meal. The cottonseed meals used in this study varied from 37 to about 58% protein. The percentage of cottonseed meal in the diet was adjusted so that the diet contained 21% protein. The

cerelose was adjusted at the same time to make up 100%. In addition to the above, the diet was supplemented with the following vitamins, in milligrams per kilogram: riboflavin 6, calcium pantothenate 15, niacin 100, vitamin B_6 3, thiamine 4, biotin 0.2, folacin 2, inositol 1,000, para-aminobenzoic acid 100, menadione 0.5, choline 2,000, and alpha-tocopherol 6. Vitamin B_{12} was also added (50 μg per kilogram). Six hundred I.U. of vitamin D_3 and 10,000 I.U. of vitamin A were added per kilogram of diet. In lysine supplementation tests, 1% DL-lysine was added.

Groups of 20 chicks each were used for testing the different meals.

Determination of nitrogen solubility in dilute sodium hydroxide

One-gram samples of cottonseed meal ground to pass a 60-mesh screen were placed in 300-ml Erlenmeyer flasks with 4 glass beads. One hundred milliliters of 0.02 normal sodium hydroxide were added and the flasks were shaken rapidly on a mechanical shaker in an incubator at 37°C. for one hour. The material was then centrifuged at 3,000 r.p.m. for 5 minutes. It was filtered through coarse filter paper if floating particles made this necessary.

Fifty milliliters of the supernatant liquid were pipetted into a Kjeldahl flask. Two milliliters of concentrated sulfuric acid were added and the material was evaporated almost to dryness on the digestion rack, after which the Kjeldahl determination for total nitrogen was carried out as usual. Nitrogen solubility is expressed as per cent of the total nitrogen which is soluble in 0.02 N sodium hydroxide.

Determination of gossypol

Free gossypol was determined according to the method of Pons and Guthrie ('49) and total gossypol by the method of Pons, Hoffpauir and O'Connor ('50).

Lysine availability

The evaluation of the cottonseed meal samples for lysine availability was conducted as described by Kuiken and Lyman ('48).

Source of meals

The cottonseed meals used in this investigation were obtained through the cooperation of the Southern Regional Research Laboratory. They were prepared under carefully controlled conditions, some in commercial mills and others in research laboratories.

RESULTS AND DISCUSSION

The results of the chick feeding tests on different types of cottonseed meals are given in table 1. In these tests cottonseed meal constituted the only source of protein, hence the growth rate was dependent on the adequacy of the protein in the samples for supplying dietary amino acid requirements. Meal 10, which was prepared by butanone extraction without heat, was chosen as a standard and assigned a growth rate index of 100. This meal has been found to have a high nutritional value in the laboratories of other collaborators where it has been tested.

It will be noted that meals which gave an excellent chick growth rate and also meals which gave a poor growth rate were found among the samples from each of the general manufacturing procedures studied.

The data indicate that the predominant factor which determines the nutritional value of the meals is total gossypol. Thus hydraulic meal 9 with a total gossypol content of 0.79% was unquestionably better than meal 25 with a total gossypol content of 1%. Among the solvent meals, standard meal 10 with a gossypol content of 0.15% was considerably better than any of the other meals, all of which contained more than 1% of total gossypol. Among the pre-press solvent meals, meal 13 had the lowest total gossypol content and the highest nutritional value. Among the screw press meals, meals

15, 16, 17, 19, and 14 all had a total gossypol content of less than 0.85%, and these meals all had a growth rate index which was higher than that of any other meal in the group.

TABLE 1

Effect of processing on the nutritional value of cottonseed meal protein

MEAL NO.	GROUP ACCORDING TO PROCESSING TYPE	GOSSYPOL		AVE. GAIN OF CHICKS AT 4 WEEKS [2]	FEED EFFICIENCY	GROWTH RATE INDEX [3]
		Free	Total			
		%	%	gm		
	Hydraulic					
9	225° [1]	0.022	0.79	170.0	3.03	79.0
25			1.00	93.0	4.23	43.2
	Solvent					
10	Standard meal (butanone-extracted, not cooked)	0.021	0.15	215.2	2.69	100.0
24			1.17	107.7	3.89	50.0
29			1.15	125.4	3.11	58.3
3	190°–200°	0.06	1.30	92.6	4.46	43.0
5	190°–210°	0.057	1.18	112.4	3.95	52.2
4	200°–226°	0.028	1.28	85.4	4.79	39.7
2	203°–224°	0.027	1.32	82.6	4.30	38.4
1	230°	0.033	1.34	84.6	4.00	39.3
	Prepress-solvent					
13	190°	0.019	0.76	208.9	2.79	97.1
11	190°	0.014	1.02	143.5	3.65	66.7
12	200°	0.038	1.11	132.1	3.67	61.4
	Screw press					
15	160°	0.05	0.63	213.3	2.74	99.1
16	180°	0.03	0.62	220.2	2.63	102.4
19	200°	0.029	0.84	144.3	3.29	67.0
22	200°	0.031	0.94	95.9	4.31	44.6
14	230°	0.03	0.66	201.9	2.78	93.8
20	230°	0.030	0.95	120.6	3.71	56.0
18	240°	0.043	0.87	118.1	3.97	54.9
23	240°	0.043	1.11	66.4	6.52	30.8
21	250°	0.04	1.11	48.8	6.66	22.7

[1] Maximum processing temperature.

[2] Average values for two assays.

[3] Growth rate index $= \frac{\text{Ave. gain of chicks at 4 weeks}}{\text{Ave. gain of chicks at 4 weeks on standard meal}} \times 100.$

The effect of total gossypol content on the nutritional value of cottonseed meal protein should not be confused with the known toxicity of free gossypol. The majority of the meals used in this investigation were very low in free gossypol, and all of them were reasonably low. In this case total gossypol is essentially bound gossypol. That bound gossypol is resistant to digestion and passes through the digestive tract in an insoluble form is clearly shown by the work of Carruth ('47), who fed rats cottonseed meal with known bound gossypol content and recovered 85% of the gossypol in the feces. It is logical to assume that if bound gossypol passes through the digestive tract in an insoluble form, fractions of the protein associated with it would also be excreted in the feces. The net result would be a reduction in the amount of amino acids available to the animal for tissue building.

Examples of the inconsistency of the results obtained in the attempt to use nitrogen solubility in dilute sodium chloride solution as a measure of protein quality are given below: Meal 9 with a growth rate index of 79.0 had a protein solubility of only 17.2%, while meal 3 with a growth rate index of 43.0 had a protein solubility of 47.6%, and meal 14 with a growth rate index of 93.8 had a protein solubility of 23.3%.

The question of the relationship of processing temperature to the nutritional value of the protein is particularly important. The data presented here indicate that relatively low processing temperatures cannot be depended on alone as a means of obtaining meals with high protein quality. Thus, meals 3, 11, 12, and 22 were all prepared with heat treatments of 200°F. or less, and yet these meals were either mediocre or poor. On the other hand, meals 9 and 14, processed at a temperature of 225° and 230°, respectively, would be classed among the better meals. It is probable that the characteristics of the seed used in the preparation of these meals were an important factor. Further tests would be needed to substantiate this hypothesis. The finding that low temperature alone cannot be depended upon to produce meals

of high protein quality is not in contradiction to the postulate that excessive heat treatment may result in meals of poor nutritional value. It may well be that the extremely low growth rate index value of 22.7 obtained from meal 21 was partially due to the fact that this meal was processed at a maximum temperature of 250°F.

Seeking a chemical characteristic which would be more adequate for measuring the nutritional value of the protein, a relationship was found between the solubility of the protein in dilute sodium hydroxide and chick growth rate (see table 2). However, a number of samples were found with high protein solubility but low growth rate values. In table 2 it will be seen that these are samples with high total gossypol content; for example, meal 3.

Having recognized two meal characteristics which are associated with chick growth, a chemical index was devised which takes both factors into consideration. The per cent nitrogen solubility in dilute sodium hydroxide is divided by the total gossypol content of the sample (expressed as per cent) except when the total gossypol content is less than 0.85%, in which case the figure 0.85 is used. It appears that when the total gossypol content is below approximately 0.8%, further reduction in total gossypol does not result in marked changes in nutritional value. The correlation between chick growth rate and the chemical index as shown in table 2 suggests that the chemical index may be a useful analytical tool in processing studies for the improvement of cottonseed meal.

In order to determine whether differences in lysine availability could account for the wide differences obtained in the chick growth rate tests, lysine availability studies with rats were conducted on representative samples of meal. The data given in table 2 show a progressive decrease in lysine availability with a decreased rate of chick growth.

These results suggest that the poorer meals might be greatly improved by lysine supplementation. The results of two different chick feeding tests on the same meals, one in which lysine was added to all rations, are given in table 3.

In a sense the values for a given meal, with and without lysine, are not strictly comparable, in that the experiments were not run concurrently. However, the data do indicate that the response to lysine supplementation is dependent on

TABLE 2

Nutritional value and chemical characteristics of cottonseed meals

MEAL NO.	TOTAL GOSSYPOL	NITROGEN SOLUBILITY, IN 0.02 N NaOH	CHICK GROWTH RATE INDEX [1]	CHEMICAL INDEX [2]	LYSINE AVAILABILITY
	%	%			
10	0.15	83.1	100.0	97.8	80.7
16	0.62	83.5	102.4	98.2	
15	0.63	87.7	99.1	103.2	82.6
13	0.76	75.9	97.1	89.3	76.9
14	0.66	69.8	93.8	82.1	
9	0.79	69.9	79.0	82.2	
19	0.84	60.7	67.0	71.5	
11	1.02	67.9	66.7	66.6	
12	1.11	64.6	61.4	58.2	
29	1.15	75.5	58.3	65.6	
20	0.95	53.7	56.0	56.5	
18	0.87	53.3	54.9	61.3	
5	1.18	71.2	52.2	60.3	
24	1.17	64.5	50.0	55.1	68.1
22	0.94	53.7	44.6	56.5	55.3
25	1.00	53.4	43.2	53.4	
3	1.30	75.3	43.0	57.1	
4	1.28	64.8	39.7	50.6	
1	1.34	57.5	39.3	42.9	
2	1.32	60.9	38.4	46.1	
23	1.11	55.8	30.8	50.3	53.2
21	1.11	50.9	22.7	45.6	50.7

[1] Average values for two assays.

[2] Chemical index $= \frac{\%\text{ nitrogen solubility in 0.02 N NaOH}}{\text{Total gossypol expressed as \%}}$.

If the total gossypol content is less than 0.85, the figure 0.85 is used instead of the gossypol content.

the condition of the protein in the meal to be tested. It will be noted that the increase in growth rate in the presence of lysine with the poorest of the meals was over 100%, while the difference with and without lysine in the case of the

better meals was fairly small. The increase in feed efficiency on the addition of lysine to the poor meals is of interest.

It is also of interest to note that supplementation of cottonseed meal with lysine increased the 4-week gain of the chicks over that obtained by feeding soybean oil meal. The interesting question is raised as to whether cottonseed meal may contain an unidentified chick growth factor when properly supplemented with lysine.

TABLE 3

The effect of lysine supplementation on the nutritional value of cottonseed meals for chicks

COTTONSEED MEAL NO.	4-WK. GAIN [1]			GRAMS GAIN/GRAMS FEED	
	Without lysine	With lysine	Per cent increase with lysine	Without lysine	With lysine
	gm	*gm*			
25	101.8	236.8	132.6	3.95	2.80
20	111.4	258.3	131.8	3.98	2.85
29	125.7	277.2	120.5	3.38	2.59
24	124.6	241.1	93.5	3.29	2.33
19	144.3	282.1	95.5	3.40	2.52
9	153.6	218.0	41.9	3.20	3.02
17	155.3	239.8	54.4	3.22	2.72
14	207.0	253.3	22.3	2.79	2.47
10	210.3	260.0	23.8	2.63	2.36
16	220.2	261.9	18.9	2.71	2.70
Soybean oil meal	212.4			2.35	

[1] Average values for 20 chicks on each meal tested.

SUMMARY

Twenty-three samples of cottonseed meals, made by different manufacturing processes, were evaluated by chick feeding tests.

Wide variations in the protein quality of the meals made by each process were found.

Low processing temperatures did not always produce high quality meals.

Although the free gossypol content of most of the meals was quite low, the total gossypol content of the meals proved to be an important factor in determining the nutritional value of the protein.

A relationship was found between the solubility of the protein in 0.02 N sodium hydroxide and the nutritional value of the meal.

A chemical index which takes into account both total gossypol content and nitrogen solubility, in 0.02 sodium hydroxide, was devised for the chemical evaluation of protein quality in cottonseed meals. A good correlation was found between the chemical index values and chick growth rate.

Lysine availability as determined by rat feeding tests proved be closely related to the values obtained for the meals by chick growth rate.

Lysine supplementation of the poorer meals resulted in an increased growth rate of chicks amounting to over 100%. Lysine supplementation of the better meals resulted in some improvement in growth rate, but the percentage increase was less.

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